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<b>(54) Title:</b> DETECTION OF HUMAN $\alpha$ -THALASSEMIA MUTATIONS AND THEIR USE AS PREDICTORS OF BLOOD-RELATED DISORDERS  <b>(57) Abstract</b>  The invention is based on the discovery that adults having a genotype comprising a hemoglobin $\alpha$ -gene deletion are significantly more likely to be hypertensive than adults having a normal ( $\alpha\alpha/\alpha\alpha$ ) genotype. The invention provides an improved method for determining a human subject's genotype at the $\alpha$ -gene loci; a method of screening a human subject for an increased potential of developing hypertension and other blood-related disorders; and provides an apparatus/kit for screening a human subject for a risk of developing hypertension and other blood-related disorders.		

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**DETECTION OF HUMAN  $\alpha$ -THALASSEMIA MUTATIONS AND  
THEIR USE AS PREDICTORS OF BLOOD-RELATED DISORDERS**

**BACKGROUND OF THE INVENTION**

**A. Field of the Invention**

5                   The present invention relates in general to a method and to a kit of materials for performing assays for blood-related disorders, and more particularly to a method and kit for predicting whether a human subject has a mild  $\alpha$ -thalassemia genotype, and whether a human subject will develop hypertension.

10    **B. Background**

                  The modern development of molecular biological techniques has permitted investigation into the genetic abnormalities that cause, or correlate with, specific human disease states and conditions. For example, restriction fragment length polymorphism (RFLP) analysis facilitates the identification of genetic defects which cause or are correlated with disease states, and facilitates the identification of individuals possessing the genetic defects. RFLP procedures involve digesting DNA with one or more restriction enzymes and analyzing the restriction fragments using, e.g., Southern blot hybridizations employing selected gene probes. See Alberts *et al.*, *Molecular Biology of the*  
15    *Cell*, Second Edition, New York: Garland Publishing (1989), pp. 270-71.

                  The polymerase chain reaction (PCR) and its many variations are particularly useful tools for investigation into genetic abnormalities that underlie disease states and conditions. (See, e.g., Erlich *et al.*, *Current Communications in Molecular Biology: Polymerase Chain Reaction*, Cold  
25    Spring Harbor: Cold Spring Harbor Press (1989); Innis *et al.*, *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press (1990).) PCR is used to amplify a DNA or one or more portions thereof that are of particular interest, to facilitate further characterization of the amplified portion. Such further characterization includes gel electrophoresis to determine size,  
30    nucleotide sequencing, hybridization studies using particular probes, and the like. See generally, Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Second Edition, Cold Spring Harbor: Cold Spring Harbor Press (1989).

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- When a genetic abnormality has been correlated with a physiological condition or disease state, Northern and Southern hybridization techniques [see Sambrook *et al.* (1989), chapters 7 and 9] with a specific oligonucleotide probe permit the determination of whether the genetic
- 5 abnormality is present or absent in a particular individual's genome. Nucleotide sequencing techniques may be used to confirm that a particular individual possesses or lacks a particular known genetic trait. See, e.g., Sambrook *et al.*, Chapter 13; Drmanac *et al.*, *Science*, 260:1649-52 (1993); Mirzabekov, *TIBTECH*, 12:27-32 (1994); Innis *et al.*, U.S. Patent No.
- 10 5,075,216.
- Hemoglobin is a complex, iron-containing, allosteric erythrocyte protein that carries oxygen from the lungs to cells and carbon dioxide from cells to the lungs. Hemoglobin A, the principle adult hemoglobin protein, comprises four polypeptide chains (two  $\alpha$ -globin chains and two  $\beta$ -globin
- 15 chains) and is among the best characterized of human proteins. *Human Hemoglobins*, H.F. Bunn, B.G. Forget, and H.M. Ranney (Eds.), Philadelphia: W.B. Saunders Co., (1977). A number of human disease states have been attributed to genetic mutations effecting one or more of the genes encoding hemoglobin polypeptide chains, including sickle cell anemia, which
- 20 results from a point mutation in the hemoglobin  $\beta$ -chain. Alpha- and beta-thalassemia conditions are blood-related disorders which result from genetic mutations manifested phenotypically by deficient synthesis of one type of globin chain, resulting in excess synthesis of the other type of globin chain. See generally Weatherall *et al.*, *The Thalassemia Syndromes*, 3rd ed.,
- 25 Oxford, Blackwell Scientific, 1981.
- Alpha-thalassemia and its associated clinical syndromes can result from either a deletion or a point mutation effecting one or both  $\alpha$ -globin genes (or gene controlling sequences) that results in reduced synthesis of hemoglobin alpha-chains. The resultant imbalance between  $\alpha$  and  $\beta$  chain
- 30 production leads to accumulation of excess  $\beta$  chains that are unstable and precipitate within the cell, altering the cell membrane and causing the clinical manifestations. Higgs, *Q. J. Med.* 86:559-64 (1993); Schrier and Mohandas, *Blood*, 79:1586-92 (1992). Greater than 95% of  $\alpha$ -thalassemia conditions are caused by deletions of one or more  $\alpha$ -globin genes, whereas structural
- 35 mutations only rarely cause a thalassemic syndrome. Kazazian, *Semin.*

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*Hematol.*, 27:209-28 (1990); Embury et al., *J. Clin. Invest.*, 66:1319-25 (1980).

In a normal individual, two  $\alpha$ -globin genes along with three other pseudogenes ( $\psi\zeta_1$ ,  $\psi\alpha_2$ , and  $\psi\alpha_1$ ) and a gene of undetermined function ( $\theta_1$ ) are all located on the short arm of chromosome 16 in the order: telomere-  
 5  $\psi\zeta_1$ - $\psi\alpha_2$ - $\psi\alpha_1$ - $\alpha_2$ - $\alpha_1$ - $\theta_1$ -centromere. See generally, Higgs et al., *Blood*, 73:1081-104 (1989). Thus, a normal individual (genotype  $\alpha\alpha/\alpha\alpha$ ) possesses four  $\alpha$ -globin genes: two on each chromosome 16.

The mild  $\alpha$ -thalassemia genotype -- deletion of one of the four  
 10  $\alpha$ -globin genes -- is one of the most common genetic abnormalities in the world. Kan, *JAMA*, 267:1532-6 (1992); Baysal and Huisman, *Am. J. Hematol.*, 46:208-13 (1994); Liebhaber, *Hemoglobin*, 13:685-731 (1989). Among black Americans, this condition occurs with a prevalence of 27.5% (Dozy et al., *Nature*, 280:605-7 (1979)), representing a gene frequency of  
 15 0.16, and suggesting that over 8 million black Americans could be affected by this condition.

The majority of these single alpha gene deletions are characterized by a  $\sim 3.7$  kilobase (kb) deletion ( $-\alpha^{3.7}$ ; "rightward deletion") within the  $\alpha$  gene locus as depicted in Fig. 1. Embury et al. (1980); Higgs et al. (1989). Three subclasses of the  $-\alpha^{3.7}$  deletion (Fig. 1,  $-\alpha^{3.7I}$ ,  $-\alpha^{3.7II}$ ,  $-\alpha^{3.7III}$ )  
 20 have been detected by Southern blot hybridization. Higgs et al., *Nucleic Acids Res.*, 12:6965-77 (1984). In all three classes, the 3.7 kb deletion results in a single, hybrid, functional  $\alpha$  gene consisting of a 5'  $\alpha_2$  sequence and a 3'  $\alpha_1$  sequence. The genotype of a heterozygous individual inheriting one  $-\alpha^{3.7}$  and  
 25 one normal chromosome 16 may be expressed as  $-\alpha^{3.7}\alpha/\alpha\alpha$ . A "leftward deletion," resulting in the loss of a 4.2 kb segment containing the  $\alpha_2$  locus ( $-\alpha^{4.2}$ ), also has been characterized. (See Fig. 1.) The  $-\alpha^{4.2}\alpha/\alpha\alpha$  genotype is rare in the overall U.S. population (Higgs et al. (1989)), but can be prevalent in 58% of some Chinese populations. Baysal and Huisman (1994).

If an  $\alpha$ -globin gene deletion is present on both of an  
 30 individual's chromosomes, a clinically observable thalassemia occurs ( $\alpha$ -thalassemia trait). This genotype occurs with an incidence of almost 3% in American blacks. Dozy et al. (1979). It is, therefore, likely to be a significant cause of microcytic anemia that does not respond to oral iron  
 35 therapy in black children. Kazazian (1990).

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In individuals from Southeast Asia and the Mediterranean areas, the most common deletions involve both  $\alpha$  genes on the same chromosome 16 (Fig. 1: --SEA or --MED). The prevalence of the --SEA mutation is 3-5 % in Southeast Asia. Bowden *et al.*, *Br. J. Haematol.*, 81:104-8 (1992). The  
5 prevalence of the --MED deletion is less than 1 % in the Mediterranean basin. *Id.* Several other  $\alpha$  gene deletions have been characterized. See, e.g., Higgs *et al.* (1989); Baysal and Huisman (1994). Inheritance of double deletions on both chromosomes (e.g. --SEA/--SEA) results in no  $\alpha$ -chain production, and such fetuses often die *in utero*. If three of an individual's  $\alpha$ -gene loci are  
10 deleted (e.g.,  $-\alpha^{3.7}\alpha/--SEA$ ), severe thalassemia occurs (hemoglobin H disease).

Since most  $\alpha$ -thalassemia mutations are due to deletions of one or more  $\alpha$ -loci on chromosome 16 (Kazazian (1990), *supra*; Embury *et al.* (1980), *supra*), specific PCR-based molecular biological techniques to detect  
15 these deletions (versus non-deletional mutations) have been developed. See, e.g., Baysal and Huisman (1994), *supra*; Bowden *et al.* (1992), *supra*; Dode *et al.*, *Br. J. Haematol.*, 76:275-81 (1990); Lebo *et al.*, *Hum. Genet.*, 85:293-9 (1990); Lundeberg *et al.*, *Biotechniques*, 10:68-75 (1991); all incorporated by reference in their entirety. While these methods take advantage of the  
20 sensitivity of polymerase chain reaction (PCR) techniques, some (Dode *et al.* (1990); Lebo *et al.* (1990)) require the use of restriction enzyme cleavage of the amplified product, prior to analysis, for adequate interpretation. In addition, none of these methods attempt to reliably and objectively distinguish between homozygotes, heterozygotes, and normals by using quantitative PCR  
25 techniques. See Lundeberg *et al.* (1991); Lubin *et al.*, *Mol. Cell. Probes*, 5:307-17 (1991). Improved techniques are needed for detecting  $\alpha$ -globin gene mutations rapidly and inexpensively, and for reliably distinguishing between various  $\alpha$ -globin genotypes.

Hypertension (high blood pressure), a prevalent and chronic  
30 cardiovascular disorder, comprises a significant risk factor for cardiovascular disease generally, and more particularly for coronary artery disease, heart failure, kidney failure, stroke, and other conditions that, collectively, afflict millions of people and consume vast amounts of society's health care resources. An estimated 15-20% of the adult population of the United States  
35 and other industrialized nations are afflicted with hypertension. See James and

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Baker, "Human Population Biology and Blood Pressure: Evolutionary and Ecological Considerations and Interpretations of Population Studies," in *Hypertension: Pathophysiology, Diagnosis, and Management. Second Edition*, Laragh and Brenner (eds.), New York: Raven Press, Ltd., Chapter 8 (1995).

5           Hypertension in certain subpopulations is even more widespread and problematic than in the population as a whole. High blood pressure is the major health problem in most black populations in the Western Hemisphere and is emerging as a major problem in many urban areas of Africa. See generally Grim *et al.*, "High Blood Pressure in Blacks: Salt, Slavery, Survival, Stress, and Racism," in *Hypertension: Pathophysiology, Diagnosis, and Management. Second Edition*, Laragh and Brenner (eds.), New York: Raven Press, Ltd., Chapter 12 (1995). The prevalence of hypertension in African Americans is the highest in the world. *Id.* Various hypotheses have been advanced to explain the prevalence of hypertension in African Americans, including the evolution of a salt-conserving genotype and the stress of living in a racist society. *Id.*

          Increasingly, individuals who are made aware of the health risks associated hypertension are able to take steps to manage their blood pressure, including lifestyle management choices (e.g., dietary choices, weight management, exercise, consumption of alcohol and tobacco). Unfortunately, hypertension generally is asymptomatic until complications develop. Consequently, many undiagnosed individuals exist who are unaware of the beneficial blood pressure management regimens in which they can engage. A long felt need exists to better identify individuals at risk for developing hypertension, so that such individuals may counseled as to lifestyle regimens which reduce the likelihood of the development of hypertension, and so that such individuals may act to monitor themselves more closely for the development of hypertension. Although certain known factors predispose individuals to hypertension (family history, weight), a long felt need exists for markers which identify individuals having a predisposition for hypertension.

          For some hypertensive patients, physicians are able to diagnose an underlying causative factor for the high blood pressure and treat the patients with the most appropriate treatment regimen. However, the vast majority of hypertensive patients suffer from "primary" (essential) hypertension, where physicians are unable to identify a particular cause for hypertension. A long

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felt need exists to identify causative factors for hypertension in individuals, to permit physicians to prescribe the most effective treatment regimens having the fewest side-effects, and to permit the development of new treatments tailored to the particular causative factors.

5                   Notwithstanding the availability of promising investigative tools such as PCR, progress in the identification of genetic markers for hypertension has been reported to be slow, and to likely be a long time in coming, due to the at least 274 genes that appear to have major influences on physiological control systems that lead to increases in blood pressure, blood sugar, obesity,  
10                   and cholesterol. Grim *et al.*, *supra*, p. 182; Leckie, *BioEssays*, 14(1):37-41 (1992).

#### **SUMMARY OF THE INVENTION**

                  The present invention solves one or more of the aforementioned needs by identifying a genetic marker useful for predicting the development of  
15                   certain blood-related disorders, including hypertension, by providing a method of screening a human subject for an increased potential (i.e., a risk) of developing a blood-related disorder, and by providing an apparatus/kit for screening a human subject for an increased potential of developing a blood-related disorder. The genetic marker can be used alone or in combination with  
20                   other genetic markers for predicting the development of a blood-related disorder. In a related aspect, the invention provides an improved method for determining a human subject's genotype with respect to a particular gene locus (i.e., the  $\alpha$ -globin gene locus on human chromosome 16).

                  The invention is based on the discovery that adults having a  
25                   genotype comprising a hemoglobin  $\alpha$ -gene deletion are significantly more likely to be hypertensive than adults having a normal ( $\alpha\alpha/\alpha\alpha$ ) genotype. Thus, in one aspect the invention provides a method of screening a human subject for a risk of developing a blood-related disorder comprising the steps of: (a) assaying genomic DNA of a human subject to determine a presence or  
30                   an absence of an  $\alpha$ -globin mutation; and (b) correlating the presence of an  $\alpha$ -globin mutation in the genomic DNA to a risk of developing a blood-related disorder. In a preferred embodiment, the method screens a human subject for a risk of developing hypertension, stroke, myocardial infarction, diabetes, hemochromatosis, and/or preeclampsia/eclampsia conditions. More



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preferably, the blood-related disorder is hypertension, stroke, myocardial infarction, diabetes, and/or hemochromatosis. Most preferably, the method screens a normotensive human subject for hypertension.

5 A person (e.g., a medical practitioner or other health care practitioner) can estimate a probability that any particular individual will develop a blood-related disorder, basing the estimate upon, e.g., the prevalence of the disorder in the general population or the individual's ethnic population, the individual's sex and age, and the like. In the present method, the phrase "screening a human subject for 'a risk' of developing a blood-  
10 related disorder" means screening a human subject for the presence or absence of a genetic marker (i.e., an  $\alpha$ -globin mutation), the presence of which correlates with a greater likelihood (i.e., probability) of developing the blood-related disorder than would otherwise be predicted (e.g., from the prevalence of the disorder in the general population or in the subject's ethnic population,  
15 the subject's sex and age, and the like).

By "genomic DNA" is meant DNA from the human subject containing at least the portion of chromosome 16 wherein the human  $\alpha$ -globin genes have been mapped. In a preferred embodiment, genomic DNA is isolated from a sample of the subject's somatic cells (e.g., blood cells).

20 By "an  $\alpha$ -globin mutation" is meant any genetic mutation affecting transcription or translation of the  $\alpha$ -genes of chromosome 16 that results in reduced synthesis of normal  $\alpha$ -globin chains, relative to the number of  $\alpha$ -globin chains synthesized in a normal ( $\alpha\alpha/\alpha\alpha$  genotype) human subject. In a preferred method, the subject's genomic DNA is assayed to determine a  
25 presence or an absence of an  $\alpha$ -globin deletion mutation. More preferably, the genomic DNA is assayed to determine a presence or an absence of an  $\alpha$ -globin deletion mutation selected from the group consisting of an  $-\alpha^{3.7}$  deletion, an --MED deletion, an --SEA deletion, an  $-\alpha^{4.2}$  deletion, and an --BRIT deletion. Of particular interest is a method of assaying the genomic DNA to determine a  
30 presence or an absence of an  $-\alpha^{3.7}$  deletion mutation, since such mutations are prevalent in, e.g., black American populations. It has been discovered that the presence of an  $-\alpha^{3.7}$  deletion correlates to a risk of developing hypertension even when the human subject is heterozygous for an  $-\alpha^{3.7}$  deletion (e.g.,  $\alpha\alpha/-\alpha^{3.7}\alpha$ ) and is free from clinical manifestations of an  $\alpha$ -thalassemia condition.

35 In another aspect, the invention comprises a method of

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determining an increased potential for developing hypertension in a normotensive human individual comprising the steps of: (a) isolating DNA from the human individual; (b) assaying the DNA for the presence of a deletion relative to DNA of a normal human subject, the deletion comprising a reduction in the number of  $\alpha$ -globin genes in the genome of the normotensive human individual; and (c) determining a potential for developing hypertension in the normotensive human individual, wherein the presence of the deletion in the DNA isolated from the normotensive human individual is indicative of an increased potential for developing hypertension. By "normotensive human individual" is meant a person whom a physician would not diagnose as hypertensive.

By "increased potential for developing hypertension" is meant a greater likelihood (probability) of developing hypertension than would be predicted (statistically) based solely upon an individual's other traits known to influence blood pressure (e.g., the individual's age, sex, ethnicity, and the like). According to the method of the invention, if a deletion comprising a reduction in the number of  $\alpha$ -globin genes is present in the DNA of a normotensive human individual, the individual is determined to have an increased potential for developing hypertension. By "reduction in the number of  $\alpha$ -globin genes" is meant a deletion which results in the human individual having fewer than the four functional  $\alpha$ -globin genes of a normal human subject.

In a preferred embodiment of the method, the DNA is assayed for the presence of a deletion of at least about 3.7 kilobases of DNA. Prevalent  $\alpha$ -globin deletion mutations such as the  $-\alpha^{3.7}$  deletion mutation, the  $-\alpha^{4.2}$  deletion mutation, the --SEA deletion mutation, and the --MED deletion mutation are deletions of at least about 3.7 kb and are deletions which comprise a reduction (by one or two genes) in the number of  $\alpha$ -globin genes in the genome, relative to the genome of a normal human subject ( $\alpha\alpha/\alpha\alpha$ ).

The skilled artisan understands that numerous molecular biological techniques may be employed to assay DNA for the presence of a deletion, particularly when the relevant portion of the human genome has been characterized extensively (as is the case for the region of chromosome 16 containing  $\alpha$ -globin genes, for which a nucleotide sequence has been determined). Thus, in one embodiment, DNA is assayed for the presence of a

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deletion by determining a partial nucleotide sequence of the subject's DNA, the partial nucleotide sequence indicating the presence or absence of the deletion. In an alternative embodiment, DNA is assayed for the presence of a deletion by performing a restriction endonuclease digestion of the subject's DNA and a Southern hybridization analysis of the restriction-digested DNA, the Southern hybridization indicating the presence or absence of the deletion. In a preferred embodiment, DNA is assayed for the presence of a deletion by performing a multiplex polymerase chain reaction as described herein in detail.

Thus, in a related aspect, the invention comprises a method for determining an increased potential for developing hypertension in a human individual comprising the steps of: (a) isolating genomic DNA from the human individual; (b) performing a multiplex polymerase chain reaction with the genomic DNA to amplify a marker portion and a control portion thereof, wherein the presence of an  $\alpha$ -globin gene deletion in the genomic DNA reduces the quantity of marker amplicons produced in the multiplex polymerase chain reaction relative to the quantity of control amplicons produced in said multiplex polymerase chain reaction; and (c) determining a potential for developing hypertension from the quantity of marker amplicons relative to the quantity of control amplicons.

More particularly, the invention comprises a method for determining an increased potential for developing hypertension in a human individual comprising the steps of: (a) isolating genomic DNA from the human individual; (b) performing a multiplex polymerase chain reaction with the genomic DNA to amplify a marker portion and a control portion thereof, wherein the presence of an  $\alpha$ -globin gene deletion in the genomic DNA reduces the quantity of marker amplicons produced in the multiplex polymerase chain reaction relative to the quantity of control amplicons produced in the multiplex polymerase chain reaction; (c) determining a ratio of marker amplicons to control amplicons produced in the multiplex polymerase chain reaction; and (d) comparing the ratio of step (c) to a ratio of marker amplicons to control amplicons produced in a multiplex polymerase chain reaction performed with genomic DNA of a normal human subject, wherein a lower marker amplicon to control amplicon ratio of step (c) indicates an increased potential for developing hypertension.

As described in detail herein, a multiplex polymerase chain

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reaction is a polymerase chain reaction wherein more than one region (segment) of template DNA is amplified simultaneously in a single reaction vessel. The amplification of more than one region of template DNA is achieved through the use of more than one pair of PCR primers, each pair of  
5 primers designed to amplify a single region of template DNA. An "amplicon" is a discreet amplification product synthesized in the M-PCR reaction and corresponding to one of the regions that is intended to be amplified.

By "marker portion" of genomic DNA is meant a segment of genomic DNA (on chromosome 16) that is amplified in the M-PCR reaction  
10 unless an  $\alpha$ -globin gene deletion is present, in which case no amplification of the segment occurs. Human individuals possess two chromosomes 16, either or both of which may have an  $\alpha$ -globin gene deletion. By "control portion" of genomic DNA is meant a segment of genomic DNA that is amplified in the M-PCR reaction irrespective of whether an  $\alpha$ -globin gene deletion is present on  
15 one or both of an individual's two chromosomes 16. Thus, the presence of an  $\alpha$ -globin gene deletion in an individual's genomic DNA reduces the quantity of marker amplicons produced in the multiplex polymerase chain reaction relative to the quantity of control amplicons produced in the multiplex polymerase chain reaction. The quantity of marker amplicons produced is reduced to  
20 essentially zero if both of an individual's chromosomes 16 contain the deletion.

In a preferred embodiment of the method, the step of performing a multiplex polymerase chain reaction includes the step of preparing a polymerase chain reaction solution, the solution comprising genomic DNA isolated from the human individual, a first pair of  
25 oligonucleotide primers for amplifying the marker portion of the DNA, and a second pair of oligonucleotide primers for amplifying the control portion of the DNA. Generally, the polymerase chain reaction solution further contains a PCR buffer, dNTP's (dATP, dGTP, dCTP, and dTTP), and a thermostable DNA polymerase enzyme. Optionally, the polymerase chain reaction solution  
30 contains more than two pairs of oligonucleotide primers (e.g., a solution may contain a third pair of primers, for amplifying a second marker portion of the DNA). A preferred pair of primers for amplifying a marker portion of DNA are primers F37.1F and F37.3, having the nucleotide sequences in SEQ ID NO: 4 and 5, respectively. A preferred pair of primers for amplifying a  
35 control portion of DNA are primers GH20F and PCO4, having the nucleotide

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sequences in SEQ ID NO: 8 and 9, respectively.

It will be apparent from the teachings herein that when M-PCR is performed using a particular first pair of primers and a particular second pair of primers, then a characteristic ratio of marker amplicons to control amplicons will result for template DNA isolated from a normal human subject (genotype  $\alpha\alpha/\alpha\alpha$ ). A characteristic, statistically-significant lower ratio will result for template DNA isolated from a heterozygous human individual having one normal chromosome 16 ( $\alpha\alpha$ ) and one chromosome 16 having an  $\alpha$ -globin gene deletion. For example, using the preferred pairs of primers described in the preceding paragraph in a series of replicate M-PCR reactions, the intra-run peak ratios (mean  $\pm$  standard deviation) in normal human subjects was  $0.790 \pm 0.081$  ( $n=6$ ). The intra-run mean for  $-\alpha^{3.7}$  deletion heterozygotes was  $0.191 \pm 0.026$  ( $n=7$ ). In the between-run replicate analysis (5 runs over 8 weeks at 1-2 week intervals), the ratio in normals was  $0.879 \pm 0.163$  ( $n=5$ ), and in  $-\alpha^{3.7}$  heterozygotes was  $0.217 \pm 0.059$  ( $n=5$ ).

It will further be apparent that a number of discrete "cut-off" ratios may be selected statistically to distinguish between "normals" and "heterozygotes" with a desired sensitivity, specificity, positive predictive value, and/or negative predictive value. Selection of a cut-off is within the ordinary skill of the art using routine statistical analysis. For the preferred pairs of primers described above, a preferred cut-off ratio of is a ratio that is about two standard deviations below (lower than) the characteristic ratio for normal human subjects, i.e., a ratio of about 0.6.

In a preferred embodiment, the method further comprises isolating genomic DNA from a normal human being; performing a control multiplex polymerase chain reaction with the genomic DNA of the normal human being to amplify a marker portion and a control portion thereof; and determining a ratio of marker amplicons to control amplicons produced in the control multiplex polymerase chain reaction; wherein in step (d), a lower marker amplicon to control amplicon ratio for the human individual compared to the normal human being indicates an increased potential for developing hypertension. The performance of these additional steps provides an "intra-run" ratio of marker amplicons to control amplicons for a normal human subject, for comparison to the ratio of amplicons determined for the human individual being tested. It is believed that the determination of such an "intra-

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run" ratio provides optimum accuracy in the method of the invention.

In a related aspect, the invention provides a genetic counseling method comprising the steps of: (a) isolating genomic DNA from a human individual; (b) assaying the DNA for a deletion relative to DNA of a normal human subject, the deletion reducing the number of  $\alpha$ -globin genes in the genome of the human individual; (c) determining a potential for developing a blood-related disorder in the human individual, wherein the presence of the deletion in the genomic DNA of the human individual is indicative of an increased potential for developing the blood-related disorder, the blood-related disorder selected from the group consisting of hypertension, stroke, myocardial infarction, diabetes, hemochromatosis, a preeclampsia condition, and eclampsia; and (d) advising the human individual with respect to the individual's potential for developing the blood-related disorder. By "advising" the human individual is meant, e.g., providing the individual with the assay results and with the interpretation of those results (i.e., either normal or an increased potential for developing the blood-related disorder). In a preferred embodiment, the blood-related disorder is hypertension, stroke, myocardial infarction and/or diabetes. In a more preferred embodiment, the blood-related disorder is hypertension.

In another related aspect, the invention provides a method of determining a hypertension-correlated genetic disorder in a hypertensive human individual comprising the steps of: (a) isolating DNA from the human individual; (b) assaying the DNA for a deletion relative to DNA of a normal human subject, the deletion comprising a reduction in the number of  $\alpha$ -globin genes in the genome of the hypertensive human individual; and (c) determining a presence of a hypertension-correlated genetic disorder in the individual from a presence of the deletion in the DNA. The determination of a hypertension-correlated genetic disorder in an individual facilitates a determination of the optimum hypertension treatment regimen for the individual.

In another aspect, the invention provides a kit for screening a human subject for an increased potential of developing a blood-related disorder, the kit comprising: an assay means for assaying a sample of genomic DNA from a human subject for the presence of an  $\alpha$ -globin mutation, wherein the assay means produces a positive  $\alpha$ -globin assay result if the  $\alpha$ -globin mutation is present in the DNA of the subject, and the assay means produces a

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negative assay result if the DNA of the subject is free of the  $\alpha$ -globin mutation; and a means for correlating a positive  $\alpha$ -globin assay result to an increased potential of developing a blood-related disorder, the disorder selected from the group consisting of hypertension, stroke, myocardial infarction, diabetes, hemochromatosis, a preeclampsia condition, and eclampsia.

Exemplary assay means include materials for performing molecular biological techniques to determine the presence of an  $\alpha$ -globin gene mutation. Thus, the assay means may include DNA sequencing materials, PCR materials, restriction digestion/southern hybridization materials, and combinations thereof. In a preferred embodiment, the assay means includes M-PCR materials. For example, the assay means comprises a pair of  $\alpha$ -globin deletion-sensitive oligonucleotide primers. The assay means may further include other materials to facilitate multiplex PCR analysis as described herein. Preferably, the assay means further comprises first and second samples of human genomic DNA, the first sample having a homozygous normal  $\alpha$ -globin genotype, the second sample having a heterozygous  $\alpha\alpha/\alpha$ -globin-deletion genotype (e.g., an  $\alpha\alpha/-\alpha^{3.7}\alpha$  genotype).

By a means for correlating a positive  $\alpha$ -globin assay result to an increased potential of developing a blood-related disorder is meant a chart, table, graph, text, or other reference that permits a medical practitioner to correlate assay results for a particular human subject to statistical risk for developing a particular blood-related disorder. For example, the chart, table, graph, or text is based on assay data disclosed herein, or upon further assay data obtained according to the teachings herein. The chart, table, graph, or text, or the data contained therein, may be provided in computer-readable form (e.g., on a floppy disc or compact disc).

In yet another aspect, the invention provides a method for assaying for a mild  $\alpha$ -thalassemia genotype in a human individual comprising the steps of: (a) isolating genomic DNA from the human individual; (b) performing a multiplex polymerase chain reaction with the genomic DNA to amplify a marker portion and a control portion thereof, wherein the presence of an  $\alpha$ -globin gene deletion in the genomic DNA reduces the quantity of marker amplicons produced in the multiplex polymerase chain reaction relative to the quantity of control amplicons produced in the multiplex polymerase chain reaction; (c) determining a ratio of marker amplicons to control

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amplicons produced in the multiplex polymerase chain reaction; and (d) comparing the ratio of step (c) to a ratio of marker amplicons to control amplicons produced in a multiplex polymerase chain reaction performed with genomic DNA of a normal human subject, wherein a mild  $\alpha$ -thalassemia genotype in the individual correlates with a lower marker amplicon to control amplicon ratio of step (c) compared to the ratio of marker amplicons to control amplicons produced in a multiplex polymerase chain reaction performed with genomic DNA of a normal human subject. Multiplex PCR as taught herein provides a characteristic ratio of marker amplicons to control amplicons for template DNA isolated from a normal human genotype; a characteristic, statistically-significant lower ratio for template DNA isolated from a heterozygote individual having one chromosome with the deletion and one chromosome without the deletion, and a ratio near zero for template DNA isolated from an individual having the deletion on both chromosomes. In preferred embodiments, the method of the invention is an assay for the presence of a  $-\alpha^{3.7}\alpha/\alpha\alpha$  genotype in a human individual. For example, a pair of M-PCR primers is employed in the M-PCR reaction to amplify a marker portion of genomic DNA that is absent from a chromosome 16 having an  $-\alpha^{3.7}$  deletion. A second pair of primers is employed to amplify a control portion of genomic DNA. Preferably, a third pair of primers also is employed to amplify a portion of genomic DNA that is present in a chromosome 16 having a  $-\alpha^{3.7}$  deletion but absent from a chromosome 16 having an --SEA, an --MED, or a --BRIT deletion. In another preferred embodiment, the method of the invention is an assay for the presence of a  $-\alpha^{4.2}\alpha/\alpha\alpha$  genotype in a human individual.

In a related aspect, the invention provides a kit for determining the  $\alpha$ -globin genotype of a human individual comprising, in association, a first pair of oligonucleotide primers for amplifying a first marker portion of a human chromosome 16, the first marker portion being absent from a human chromosome 16 having a single  $\alpha$ -gene deletion selected from the group consisting of an  $-\alpha^{3.7}$  deletion and a  $-\alpha^{4.2}$  deletion; a second pair of oligonucleotide primers for amplifying a second marker portion of a human chromosome 16, the second marker portion being present in a human chromosome 16 having the single  $\alpha$ -gene deletion and being absent from a human chromosome 16 having an --MED deletion; and a control pair of



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oligonucleotide primers for amplifying a control portion of human genomic DNA. In one embodiment, the single  $\alpha$ -gene deletion is an  $-\alpha^{3.7}$  deletion. A preferred first pair of oligonucleotide primers in this embodiment have the nucleotide sequences set forth in SEQ ID NO: 4 and 5. In another

5 embodiment, the single  $\alpha$ -gene deletion is an  $-\alpha^{4.2}$  deletion. A preferred first pair of oligonucleotide primers in this embodiment have the nucleotide sequences set forth in SEQ ID NO: 12 and 13. In either of the aforementioned embodiments, a preferred second pair of oligonucleotide primers have the nucleotide sequences set forth in SEQ ID NO: 6 and 7. By

10 "control portion" of genomic DNA is meant a segment of genomic DNA that is amplified in an M-PCR reaction irrespective of whether an  $\alpha$ -globin gene deletion is present on one or both of an individual's two chromosomes 16. A preferred control pair of oligonucleotide primers have the nucleotide sequences set forth in SEQ ID NO: 8 and 9. The kit may further include other materials

15 to facilitate multiplex polymerase chain reaction analysis as described herein. Such other materials include, for example, a PCR buffer, dNTPs, a thermostable DNA polymerase, a sample of genomic DNA having a homozygous normal  $\alpha$ -globin genotype, a sample of genomic DNA having a heterozygous  $\alpha\alpha/\alpha$ -globin-deletion genotype (e.g., a  $\alpha\alpha/-\alpha^{3.7}\alpha$  genotype or a

20  $\alpha\alpha/-\alpha^{4.2}\alpha$  genotype), combinations of such materials, and the like.

Numerous other aspects and advantages of the present invention will be apparent upon the following detailed description thereof, reference being made to the drawing wherein:

### **BRIEF DESCRIPTION OF THE DRAWING**

25 FIG. 1 is a partial map of a portion of chromosome 16 (top line), depicting the relationship between the genes which encode the alpha globin polypeptide chains ( $\alpha 1$  and  $\alpha 2$ ), common  $\alpha$ -globin gene deletions, and the deletion-sensitive primer binding regions. Each alpha gene has three *exons* (top line, black bars) separated by two *introns* (white bars). The location of

30 common  $\alpha$ -globin deletion mutations are depicted as thick black bars below the line depicting chromosome 16. The dashed lines at the ends of the black bars indicate the region in which cross-over occurs, resulting in heterogeneity with regard to the exact length of these deletions. The three related  $-\alpha^{3.7}$  deletions are depicted, each of which results in a single hybrid  $\alpha$  gene composed of a 5'

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$\alpha 2$  sequence and a 3'  $\alpha 1$  sequence. The cross-hatched boxes (above the line depicting the  $\alpha$ -globin genes on chromosome 16) represent the location of amplification products (amplicons) resulting from the binding of deletion-sensitive PCR primers. The numbers above the amplicons represent the length of the amplified product in base pairs.

FIG. 2 graphically depicts the results of a fluorescent scan of a 6% polyacrylamide gel containing fluorescein-labelled PCR amplification products (amplicons). Lane 1 (top) of the gel was loaded with a 100-bp size ladder; Lanes 2, 3, and 4 were loaded with the PCR reaction products obtained using primer pairs F13F/F14, F37.1F/F37.3, and GH20F/PCO4 (bottom), respectively, and human placental DNA as the PCR template DNA.

FIG. 3 graphically depicts the results of a fluorescent scan of a 6% polyacrylamide gel containing fluorescein-labelled multiplex PCR products wherein human placental DNA was employed as the template. Lanes 1, 2, 3, and 4 of the gel were loaded with the PCR reaction products of PCR reactions that initially contained 10, 20, 40, and 80 ng/ $\mu$ L of template DNA. Lane 5 was loaded with a 100-bp size ladder.

FIG. 4 graphically depicts the results of a fluorescent scan of a 6% polyacrylamide gel containing fluorescein-labelled multiplex PCR products wherein human leukocyte DNA was employed as the template. Lane 1 (top) was loaded with a 100 bp size ladder. Lanes 2-6 were loaded with PCR reaction products of reactions that contained template DNA from subjects of known genotypes: Lane 2,  $\alpha\alpha/\alpha\alpha$ ; Lane 3,  $\alpha\alpha/-\alpha^{3.7}\alpha$ ; Lane 4,  $-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$ ; Lane 5,  $-\alpha^{3.7}\alpha/--SEA$ ; and Lane 6,  $-\alpha^{3.7}\alpha/--MED$ .

## DETAILED DESCRIPTION

The present invention is based upon the discovery that adult subjects having a genotype comprising an  $\alpha$ -globin deletion, including subjects having a "silent carrier"  $\alpha$ -thalassemia genotype wherein only one of the four  $\alpha$ -globin genes is deleted (e.g.,  $-\alpha^{3.7}\alpha/\alpha\alpha$  genotype), are significantly more likely to suffer from hypertension than "normal" subjects ( $\alpha\alpha/\alpha\alpha$  genotype).

With reference to a particular human subject, a diagnosis of "hypertension" means that the subject has a blood pressure that is higher, to a clinically significant extent, than would be expected for a typical human subject of the same sex and age as the particular subject. Such a diagnosis is

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within the routine skill of a subject's medical practitioner, notwithstanding the fact that blood pressure (like height, weight, and other physical characteristics) is a continuous variable in a given population. A diastolic blood pressure greater than 95 mm Hg was selected to define hypertension for some human  
5 subjects in the Examples herein.

The absence (deletion) of one or more  $\alpha$ -globin genes in a subject's genome results in the synthesis of fewer than the optimum quantity of hemoglobin  $\alpha$ -chains, resulting in the existence of excess hemoglobin  $\beta$ -chains in the subject's erythrocytes. See, e.g., Kazazian (1990), *supra*; Higgs et al.  
10 (1989), *supra*. Without intending to be limited to a particular theory of the invention, it is believed that the excess  $\beta$ -chains increase the viscosity of the subject's blood relative to the blood of non-carriers (persons having a normal  $\alpha$ -globin genotype), perhaps by increasing erythrocyte cell membrane rigidity. Compare Crowley et al., *Annals Clin. Lab. Sci.*, 22(4):229-35 (1992),  
15 reporting increased blood viscosity in  $\beta$  thalassemic subjects relative to healthy subjects having similar hematocrit levels. The increased blood viscosity, in turn, contributes to hypertension.

Alternatively, it is believed that the presence of free hemoglobin in individuals having an  $\alpha$ -globin deletion genotype contributes to hypertension  
20 by binding nitric oxide (a natural vasodilator), thereby inhibiting normal vasodilation. More particularly, the excess hemoglobin  $\beta$ -chains in the erythrocytes of these individuals form  $\beta$ -chain tetramers which precipitate within the erythrocytes. The interaction between these excess  $\beta$ -chains and the erythrocyte membrane is believed to cause hemoglobin leakage from the  
25 erythrocytes into the plasma. More significantly, the excess  $\beta$ -chains in circulating erythrocytes are believed to alter the erythrocyte membrane itself (e.g., altering membrane rigidity). The altered erythrocytes are more readily destroyed in the spleen, and much of the hemoglobin from these destroyed cells is released into the lymphatic system. The increased plasma hemoglobin  
30 and, more significantly, the increased free hemoglobin in the lymph (which bathes the vascular endothelium) bind nitric oxide. The bound nitric oxide is thereby prevented from effecting vasodilation through interaction with the smooth muscles of blood vessel walls. In a related effect in the vascular compartment, the altered erythrocytes may adhere to endothelial surfaces more  
35 than normal erythrocytes, and hemoglobin in these adhering cells binds nitric

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oxide produced by the endothelial cells in close proximity. It is not believed that these theories are mutually exclusive.

Under the theory of the invention, it is believed that the physiological  $\alpha$ -chain/ $\beta$ -chain imbalance associated with the mutation of a globin gene, and more particularly with a deletion mutation affecting an  $\alpha$ -globin gene, predisposes a person to a number of additional blood-related disorders (in addition to hypertension) not previously correlated with mutations in globin genes generally or with mutations affecting  $\alpha$ -globin genes in particular.

For example, an  $\alpha$ -globin deletion genotype is believed to predispose an individual to a type of hemochromatosis, a disorder of iron metabolism characterized by excess deposition of iron in the tissues. Under the theory of the invention described above, the leakage of hemoglobin from erythrocytes (and/or the increased plasma and lymph concentrations of hemoglobin resulting from erythrocyte death and destruction) increase an individual's risk of iron overload from iron ingestion. Thus, an  $\alpha$ -globin deletion genotype causes or predisposes an individual to develop a type of hemochromatosis.

An  $\alpha$ -globin deletion genotype is believed to correlate with an increased likelihood that an individual will develop myocardial infarction or stroke. The increased blood viscosity and the reduced nitric oxide levels resulting from the damaged erythrocytes predispose individuals to develop a myocardial infarction or stroke. In the presence of atherosclerosis, the impact is even more dramatic since atherosclerotic plaques are also believed to bind nitric oxide. Since this further reduces the amount of nitric oxide available for dilatation of vascular smooth muscle, the presence of an  $\alpha$ -globin deletion genotype may result in even earlier development of myocardial infarction or stroke.

An  $\alpha$ -globin deletion genotype also is believed to predispose pregnant women to a preeclampsia condition -- a toxemia of late pregnancy characterized by hypertension, edema, and proteinuria -- and to eclampsia. Under the theory of the invention described above, erythrocyte adherence to the vascular endothelium and the increased plasma and lymph concentrations of hemoglobin resulting from erythrocyte death and destruction contribute to metabolic disturbances resulting in hypertension and other symptoms similar to

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those seen in preeclampsia, thereby predisposing an individual to develop a preeclampsia/eclampsia condition.

Similarly, an  $\alpha$ -globin deletion genotype is believed to predispose individuals to diabetes and/or to increase their risk for early expression or more severe expression of diabetic symptoms.

The determination of whether a particular human subject has a genotype comprising an  $\alpha$ -globin gene mutation, and especially an  $\alpha$ -globin gene deletion, may be performed by any procedure known in the art for genotype characterizations. Such procedures include, for example, Southern hybridization procedures, Northern hybridization procedures, RFLP procedures, PCR procedures, DNA sequencing procedures, immunological, electrophoretic, and chromatographic procedures for detection of mutant polypeptides or  $\beta$ -chain tetramers, and other procedures. Many of the foregoing procedures generally involve an analysis of isolated genomic DNA from the human subject. Any known DNA isolation procedure may be employed. In a preferred embodiment, the genomic DNA is isolated from a blood sample taken from the subject.

In one preferred embodiment, a determination of the subject's genotype is performed with a DNA sequencing procedure, since such procedures are among the most definitive methods for determining a genotype. In a highly preferred embodiment, a polymerase chain reaction procedure is performed to determine a subject's genotype, such procedures being more rapid and cost-effective than DNA sequencing procedures. See, e.g., Baysal and Huisman (1994), *supra*. Optionally, a genotype determined with a PCR procedure is confirmed using a DNA sequencing procedure. Dode *et al.* (1990), *supra*, describes a method for characterizing  $\alpha$  genes employing both PCR and sequencing. In a highly preferred embodiment, a human subject's genotype is determined after amplification of the affected region by a multiplex polymerase chain reaction (M-PCR) procedure as described herein. Optionally, the determination based upon analysis of the M-PCR reaction products is confirmed using a DNA sequencing procedure.

The present invention is further illustrated by the following examples. More particularly, Example 1 describes a multiplex polymerase chain reaction procedure for assaying (characterizing) the genomic DNA of an individual, to determine the presence or absence of a hemoglobin alpha-chain

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gene deletion in the individual's genome. Example 2 describes the use of the M-PCR procedure of Example 1 to demonstrate a correlation between a hemoglobin  $\alpha$ -chain gene deletion and the blood-related disorder hypertension in adults, thereby demonstrating that the M-PCR procedure is useful for predicting a potential of a normotensive human subject for developing hypertension.

Example 3 describes a modification of the M-PCR procedure of Example 1 useful for detecting the presence of an  $-\alpha^{4,2}$  deletion in an individual's genome. Example 4 is a prospective example which provides an M-PCR protocol for predicting a potential of a human subject for developing a blood-related disorder.

### **Example 1**

#### **Detection of common $\alpha$ -globin gene deletions with multiplex PCR**

The following procedure demonstrates a multiplex PCR procedure useful for determining the existence of an  $\alpha$ -globin gene deletion in a subject's genome. The procedure also is described in detail in Bowie *et al.*, "Detection of  $\alpha$ -Thalassemias by Multiplex Polymerase Chain Reaction," *Clin. Chem.*, 40(12):2260-66 (1994), incorporated herein by reference.

#### **Isolation of Template DNA**

DNA for use as PCR template DNA was isolated from the leukocytes of human subjects using a modification of the technique described by Lewin and Stewart-Haynes, *Biotechniques*, 13:522-3 (1992), incorporated herein by reference. EDTA-anticoagulated whole blood samples (10  $\mu$ L) from clinical specimens collected for hematological analyses were hemolyzed with 500  $\mu$ L of distilled water and centrifuged. The resultant pellet was washed twice with 500  $\mu$ L of distilled water, resuspended in 10  $\mu$ L of 0.2 M KOH containing 0.05 M dithiothreitol (DTT), and incubated at 65°C for 5 minutes. Thereafter, 1  $\mu$ L of 1.0 M HCl was added. After mixing, 10  $\mu$ L of 0.6 M Tris-HCl, pH 8.3, containing 0.2 M KCl was added. Finally, 20  $\mu$ L of sterile distilled water was added and the solution was heated at 99.9°C for 8 minutes to destroy any residual nucleases. DNA concentration was determined by absorbance measurements at 260 nm (1 absorbance unit = 50  $\mu$ g/ml of double-stranded DNA). DNA purity was assayed by absorbance measurements

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taken at 260 and 280 nm. While a 260 nm/280 nm absorbance ratio of as low as 1.0 may be indicative of sufficient purity, it is preferred that template DNA be sufficiently pure to produce a 260/280 absorbance ratio of at least 1.6. It is contemplated that DNA isolated from any other cells (e.g., from tissue sample cells) is equally suitable for use as template DNA.

#### Primer synthesis

Primer sequences were selected using known characteristics of human  $\alpha$ -globin genes, and oligonucleotide primers were synthesized for multiplex PCR as follows.

Figure 1 presents a partial map of human chromosome 16, setting forth the relative positions of the  $\alpha$ -globin genes and related gene loci. DNA sequence information for this region is known in the art [GENBANK locus HUMHBA4, "Human alpha globin psi-alpha-1, alpha-2 and alpha-1 genes, complete cds." Accessions J00153, J00079, J00154, J00155, J00156, K00418, X00168] and is set forth in SEQ ID NO: 1. The  $\alpha_2$  and  $\alpha_1$  polypeptide amino acid sequences are set forth in SEQ ID NO: 2 and 3, respectively. The regions of frequent gene deletions for the  $\alpha_1$  and  $\alpha_2$  loci also are depicted in Figure 1. Based on the published DNA sequence for this region of chromosome 16, synthetic oligonucleotide primers were designed and synthesized to be complimentary to regions of human chromosome 16 affected by common  $\alpha$ -globin gene deletions. More particularly, the nucleotide sequences of potential primer pairs were evaluated for maximum specificity, optimal GC content, and freedom from intramolecular structures using commercial software (OLIGO™, National Biosciences, Plymouth, MN). The primers were synthesized using the phosphoroamidite method on a DNA synthesizer (Gene Assembler Plus™, Pharmacia Biotechnology, Inc., Piscataway, NJ), de-protected by treatment with concentrated ammonia for one hour at 55°C, passed through NAP 10 spin columns (Pharmacia Biotechnology, Inc.), and resuspended in de-ionized H<sub>2</sub>O. The absorbances of primer solutions were read at 260 and 280 nm and diluted to an absorbance of 3.0 A (approximately 1.0  $\mu$ mol/L).

Two primers were synthesized to amplify a 375 basepair (bp) region (Fig. 1) between the  $\alpha_1$  and  $\alpha_2$  genes of a normal chromosome 16 that is absent in a chromosome 16 harboring an  $-\alpha^{3.7}$  deletion,  $--SEA$  deletion, or  $--$

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**MED deletion:**

**Primer F37.1F (SEQ ID NO: 4): 5'-FACCATCCCCCAGAAAAACAT-3'**

**Primer F37.3 (SEQ ID NO: 5): 5'-CTCTAACCATCACACAAGTA-3'**

- 5     Primer F37.1F is complimentary to the non-coding stand and corresponds to nucleotides 9166 to 9185 of SEQ ID NO: 1; Primer F37.3 is complimentary to nucleotides 9540 to 9521 of SEQ ID NO: 1.

10             A second pair of primers was synthesized to amplify a 248 bp region downstream from the  $\alpha_1$  gene of a normal chromosome 16. This 248 bp region also is present in a chromosome harboring a  $-\alpha^{3.7}$  deletion but absent from a chromosome harboring a --SEA deletion or a --MED deletion:

**Primer F13F (SEQ ID NO: 6): 5'-FACTGGAGAGGAGAGCGGGGC-3'**

**Primer F14 (SEQ ID NO: 7): 5'-AGGCTGCGGGAAGGACATCA-3'**

- 15     Primer F13F is complimentary to the non-coding stand and corresponds to nucleotides 11998 to 12017 of SEQ ID NO: 1; Primer F14 is complimentary to nucleotides 12245 to 12226 of SEQ ID NO: 1.

20             In addition to the two primer sets for detecting the aforementioned  $\alpha$ -globin gene deletions, a third pair of primers was employed to amplify an unrelated region of a subject's DNA, to serve as a positive control for the amplification reaction. A 268 bp region surrounding the 5' end of the  $\beta$  globin gene (located on chromosome 11) was selected for amplification as the positive control, using the following primers purchased from Perkin-Elmer Cetus (Norwalk, CT):

- 25     Primer GH20F (SEQ ID NO: 8): 5'-FGAAGAGCCAAGGACAGGTAC-3'

Primer PCO4 (SEQ ID NO: 9): 5'-CAACTTCATCCACGTTTACC-3'

30     The relevant portion of the  $\beta$ -globin gene locus, which is known in the art, is set forth in SEQ ID NO: 10. [See GENBANK locus HUMHBB, "Human beta globin region on chromosome 11." Accessions U01317, J00179, J00093, J00094, J00096, J00158 - J00175, J00177, J00178, K01239, K01890, K02544, M18047, M19067, M24868, M24886, X00423, X00424, and X00672.] The



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$\beta$ -chain amino acid sequence is set forth in SEQ ID NO: 11. Primer GH20F is complimentary to the non-coding strand and corresponds to nucleotides 492 to 511 of SEQ ID NO: 10; Primer PCO4 is complimentary to nucleotides 759 to 740 of SEQ ID NO: 10. These primers are known to generate a 268 bp PCR product comprising ~200 bp of DNA upstream (5') of exon 1 of the hemoglobin  $\beta$ -chain gene and extending through most of exon 1. See Bauer *et al.*, *JAMA*, 265:472-7 (1991). This segment of the  $\beta$ -globin genetic locus was chosen as the positive control because it is not the site of frequent deletions; it has been used in a variety of amplification techniques and applications as a control for amplification efficiency. See, e.g., Bauer *et al.* (1991); Dode *et al.* (1990), *supra*. The skilled artisan understands that other genetic loci that are not the site of frequent deletions also are useful controls for amplification efficiency.

The first of each pair of primers (Primer F13F, Primer F37.1F, and Primer GH20F) was labelled with fluorescein (F) at its 5' end (using fluoroscein amidite) to permit detection and analysis of PCR products obtained therefrom. (FluoroPrime™ kit, Pharmacia.) Other suitable fluorophore labels and radioisotopic labels will be apparent to those in the art.

#### Polymerase Chain Reaction

A Gene Amp™ PCR System 9600 thermal cycler from Perkin-Elmer Cetus (Norwalk, CT) was employed for all PCR amplifications. The PCR amplifications were performed with a stock (10X) PCR amplification buffer containing: 500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl<sub>2</sub>, and 0.1 g/L of gelatin, pH 8.3. Each 20  $\mu$ L amplification reaction mixture contained PCR amplification buffer (1X), 0.2 pmol of each selected primer, 2.0 nmol dNTP's (dATP, dCTP, dGTP, and dTTP, from Pharmacia Biotechnology (Piscataway, NJ)), DNA template (200-1600 ng), and 0.25U of recombinant Taq polymerase (Perkin-Elmer Cetus). No overlaying the reactions with oil was necessary.

The cycling sequence was as follows: (1) DNA denaturation at 94°C for 3 minutes; (2) 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds; (3) a final extension at 72°C for 3 minutes; and (4) cooling at 4°C. Samples were held at 4°C on the thermal cycler or in a refrigerator until analyzed as

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described below.

A blank amplification reaction containing all reagents but without template DNA was used as a negative control. As a positive control, each set of PCR tubes included a sample of human placental DNA (ONCOR, Gaithersburg, MD) or known normal ( $\alpha\alpha/\alpha\alpha$ ) human leukocyte DNA.

The optimal reaction conditions for multiplex polymerase chain reaction (M-PCR), in which two or all three sets of primers were employed in a single PCR reaction, were found to be the same as those described above.

#### Fragment Analysis and Quantitation

PCR products were analyzed using electrophoresis on a 2% agarose gel (Metaphor™, FMC BioProducts, Rockland, ME) with TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) containing 0.5 mg/L of ethidium bromide for visualization of DNA. A 6  $\mu$ L sample from a PCR reaction mixture was mixed with 2  $\mu$ L of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% sucrose in water) and run on agarose gel at 100 V for 45 minutes at room temperature. The gel was viewed under UV illumination and photographed.

To measure the relative amounts of M-PCR amplification products (amplicons) in a quantitative manner, the M-PCR reaction products were separated by electrophoresis on a 6% polyacrylamide gel using a DNA sequencer (ALF™, Pharmacia Biotechnology, Inc.). Electrophoretic conditions were 1500 volts and 34 watts. The gel was thermostated at 42°C. Samples (1  $\mu$ L PCR reaction mixture and 1  $\mu$ L stop solution (deionized formamide/Blue Dextran; Pharmacia Biotechnology)) were denatured at 94°C for 3 min. prior to loading on the gel.

The fluorescein-labelled primers permitted quantitative detection of fluorescent, amplified PCR products from the M-PCR reactions. Fluorescence was measured using a laser power of 3 milliwatts, and fluorescence peak areas were determined by integration using fragment analysis software (Fragment Manager™, Pharmacia). The ratios of the 248 and 375 bp amplicon peak areas to the 268 bp control amplicon peak area were determined in this manner.

Fragment sizes of the M-PCR amplicons were confirmed using a 100 bp size DNA ladder (Pharmacia Biotechnology, Inc.) in one of the lanes

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of the sequencing gels. The DNA ladder was labeled with fluorescein by treatment with fluorescein-labeled 12-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN). This was accomplished by adding 2  $\mu$ L each of DNA ladder (1  $\mu$ g/ $\mu$ L), Fill-In Buffer (200 mM Tris, pH 7.6, 500 mM KCl, 100 mM, MgSO<sub>4</sub>, 40 mM MgCl<sub>2</sub>, 0.5 g/L bovine serum albumin), and 1 mM fluorescein-12-dUTP to 1  $\mu$ L (1U/ $\mu$ L) Klenow Fragment (Pharmacia) and 13  $\mu$ L of distilled water. The mixture was incubated for 90 minutes at room temperature and the reaction was stopped with 2  $\mu$ L of 0.4 M EDTA. The labeled DNA was precipitated by adding 2.5  $\mu$ L of 4 M LiCl and 15  $\mu$ L of pre-chilled (-20°C) absolute ethanol and freezing for 30 minutes at -70°C. The precipitated DNA was centrifuged for 20 minutes at 1200 X g, washed with cold 70% ethanol and dissolved in 50  $\mu$ L TE buffer. This solution is stable for at least 6 months when stored at -70°C.

#### Sequencing of PCR Products

To confirm that the PCR amplified products (amplicons) were the result of the binding of the primers to the intended region, the DNA sequence of each amplicon was determined. The individual amplified products were purified from agarose gels following electrophoresis by adsorption onto glass beads (Sephaglas Band Prep Kit, Pharmacia Biotechnology, Inc.). The yield and purity of the amplicons were determined qualitatively by visually comparing the fluorescence of ethidium bromide stained bands to that of a standard DNA marker (e.g., a DNA fragment size ladder) and quantitatively by determining the ratio of absorbances at 260 nm and 280 nm of samples and standards by spectrophotometry. The DNA cycle sequencing protocol was essentially that recommended in the fmol™ DNA Sequencing System (Promega, Madison, WI), except for the substitution of fluorescein-labeled primers (1.0  $\mu$ mol/L) for radio-labeled primers. DNA sequence determinations were performed using the ALF™ DNA Sequencer (Pharmacia Biotechnology, Inc.).

#### Results

In one set of PCR reaction mixtures, the primer pairs described above were employed individually (one primer pair per reaction tube) to amplify human placental template DNA (40 ng/ $\mu$ L, i.e., ~ 800 ng/reaction).

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PCR was performed and the reaction products analyzed as described above.

Agarose gel electrophoresis of the reaction products revealed a single prominent band for each reaction, the size of which was consistent with the amplification product predicted for a given reaction (~248 bp for primers F13F and F14; ~375 bp for primers F37.1F and F37.3; and ~268 bp for primers GH20F and PCO4).

The identity of the PCR products was further confirmed with polyacrylamide gel electrophoresis. As depicted in Fig. 2, each reaction yielded an amplification product of the size predicted for the particular primer pair employed. Early eluting peaks (100-200 bp) represent non-specific amplification products which do not affect the quantitation of target peak areas. The identity of the amplicons also was confirmed by isolation of the individual DNA bands from the agarose gel and sequencing the DNA using a cycle sequencing protocol on the ALF™ DNA Sequencer. These results with the human placental template DNA confirmed the viability of the primers for amplification of the selected 248 bp and 375 bp regions of chromosomes 16 (the  $\alpha$  gene loci) and the 268 bp region of chromosome 11 (the  $\beta$  gene locus).

Multiplex PCR reaction mixtures were prepared containing all three of the above-described primer pairs and varying concentrations of human placental template DNA (10, 20, 40, and 80 ng/ $\mu$ L, i.e., 200, 400, 800, and 1600 ng/reaction). PCR was performed and the reaction products analyzed as described above. Eight reactions were performed for each template concentration.

As predicted, agarose gel electrophoresis of the reaction products revealed three prominent bands of ~248 bp, ~375 bp, and ~268 bp. The samples were further analyzed on polyacrylamide gels, which permitted relative quantification of each amplicon band by measurement of the area of fluorescence peaks (Fig. 3). The ratio of  $\alpha$ -gene deletion-sensitive amplicon peak areas (248 and 375 bp products) to the area of the control peak (268 bp product) are summarized in Table 1 (expressed as mean values for eight samples, plus or minus one standard deviation).

**TABLE 1**  
**CONCENTRATION DEPENDENCE OF PEAK AREA RATIOS**

5	TEMPLATE DNA	<u>PEAK AREA RATIO</u>	
	CONCENTRATION	248/268 (n = 8)	375/268 (n = 8)
	<u>(ng/<math>\mu</math>L)</u>		
	10	0.79 $\pm$ 0.14	0.92 $\pm$ 0.27
	20	0.68 $\pm$ 0.03	1.00 $\pm$ 0.03
	40	0.65 $\pm$ 0.07	0.87 $\pm$ 0.08
10	80	0.60 $\pm$ 0.06	0.95 $\pm$ 0.05

The ratios of the amplified products to the control were relatively constant, but the reproducibility was deemed unacceptable (coefficient of variation greater than 10%) at initial template DNA concentrations below 20 ng/ $\mu$ L. The presence of the background peaks at peak sizes other than 248, 268, and 375 do not affect the quantitation of the peaks of interest, and these background peaks have not been further characterized. The reproducible peak area ratios indicated the viability of multiplex PCR with these three primer pairs.

To demonstrate the viability of the multiplex PCR procedure for detecting  $\alpha$ -globin gene deletions in human subjects, additional multiplex PCR reaction mixtures were prepared containing the three primer pairs and containing human leukocyte template DNA, prepared from the blood of fifteen human subjects as described above. To determine the ability of this M-PCR protocol to detect  $\alpha$ -globin gene deletions, the genotype of each subject was also determined independently via Southern blot hybridization studies using methods known in the art. (See Sambrook et al., *supra*.) For example, genomic DNA from a subject is digested with the restriction endonucleases Bam HI, Eco RI, and Bgl II, electrophoresed, blotted, and probed with a radioactively-labelled probe specific for  $\alpha$ -chain gene sequences. A preferred  $\alpha$ -chain gene probe is ATCC clone pJW101 (ATCC Accession No. 57601, *ATCC/NIH Repository Catalog of Human and Mouse DNA Probes and Libraries*, Seventh Ed., (1993)). A suitable control probe is ATCC clone pBRZ (ATCC Accession No. 57599).

M-PCR was performed and the reaction products were analyzed on agarose and polyacrylamide gels as described above. Genotypes, peak area measurements, and peak area ratios determined for each subject are

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summarized in Table 2. Fluorescence scanning measurements for selected subjects are depicted in Fig. 4.

TABLE 2 - CORRELATION OF GENOTYPES AND M-PCR PEAK MEASUREMENTS						
AREAS AND AREA RATIOS FOR AMPLIFIED PRODUCTS						
Patient Number	Genotype	Peak Area			Peak Area Ratio	
		248 bp peak	268 bp peak	375 bp peak	248/268	375/268
1	$-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$	QNS*	6096	--	QNS	--
2	$(\alpha\alpha/\alpha\alpha)/(-\beta/\beta)$	1313	10133	5032	0.13	0.50
3	$-\alpha^{3.7}\alpha/\alpha\alpha$	QNS	5879	1093	QNS	0.19
4	$(\alpha\alpha/\alpha\alpha)/(\beta\beta/\beta\beta)$	3901	13232	9665	0.29	0.73
5	$\alpha\alpha/--SEA$	QNS	2003	504	QNS	0.25
6	$\alpha\alpha\alpha/\alpha\alpha$	2000	12265	6111	0.16	0.50
7	$(\alpha\alpha/--SEA)/(\beta\beta/\beta\beta)$	457	10261	3269	0.04	0.32
8	$-\alpha^{3.7}\alpha/-\alpha^{4.2}\alpha$	QNS	7719	1778	QNS	0.23
9	$\alpha\alpha/\alpha\alpha$	816	10176	5319	0.08	0.52
10	$-\alpha^{3.7}\alpha/--SEA$	464	12323	--	0.04	--
11	$-\alpha^{3.7}\alpha/--MED$	334	12145	--	0.03	--
12	$-\alpha^{4.2}\alpha/\alpha\alpha$	5293	13221	9820	0.40	0.74
13	$(\alpha\alpha/--BRIT)/(-\beta/\beta)$	445	13866	1184	0.03	0.10
14	$\alpha\alpha/\alpha\alpha$	2322	13561	9624	0.17	0.71
15	$-\alpha^{4.2}\alpha/--SEA$	494	12655	3222	0.04	0.25

\* QNS, quantity not sufficient for reliable analysis.

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The results summarized in Table 2 and Fig. 4 demonstrate the correlation between an  $-\alpha^{3.7}$  deletion genotype and a reduced 375/268 peak area. For example, the 375/268 peak ratio of subject 3 (heterozygous for the  $-\alpha^{3.7}$  deletion; peak ratio of 0.19) was significantly reduced compared to that of subject number 9 (normal genotype; peak ratio of 0.52). Deletions of the same locus on both of a subject's chromosomes results in the complete absence of the corresponding deletion-sensitive PCR amplicon. (See, e.g., Fig. 4, lanes 4-6.) Both the 375/268 and 248/268 ratios are reduced in subjects having a --SEA or --MEA deletion on one chromosome.

Only eleven of the fifteen samples had sufficient DNA for reliable quantitation of the 248/268 area ratios for detecting --SEA and --MED deletions. This shortcoming was attributed to the fact that the amplification efficiency of the --SEA/--MED locus is significantly lower for whole blood DNA than for purified placental DNA (Fig. 4). The peak ratios for the eleven samples were consistent with the genotypes of the samples.

The differences observed in peak heights and areas between Figs. 3 and 4 probably reflect different amplification efficiencies between placental DNA and that of adult subjects, since all of the primers were in the M-PCR reaction at equal concentrations. Varying the relative concentration of primer pairs resulted in more even peak areas, but this also resulted in slightly poorer precision.

In a preferred embodiment for resolving all three peaks satisfactorily, the M-PCR is performed using a reaction mixture containing approximately 0.4 pmol of each primer for amplifying the 375 and 248 bp deletion-sensitive regions, and approximately 0.2 pmol of primers GH20F and PCO4 for amplifying the 268 bp control region.

The intra-run and between-run reproducibility of the ratios for Southern blot-confirmed normal and heterozygote genotypes were determined by replicate analysis. The intra-run peak ratios (mean  $\pm$  standard deviation) for the 248/268 and the 375/268 ratios in normals ( $\alpha\alpha/\alpha\alpha$ ) was  $0.200 \pm 0.032$  and  $0.790 \pm 0.081$  (n=6). The intra-run mean for the 248/268 and 375/268 ratios in  $-\alpha^{3.7}$  heterozygotes were  $0.201 \pm 0.025$  and  $0.191 \pm 0.026$  (n=7). In the between-run replicate analysis (5 runs over 8 weeks at 1-2 week intervals), the 248/268 and the 375/268 ratios in normals was  $0.303 \pm 0.067$  and  $0.879 \pm 0.163$  (n=5). The between-run mean for the 248/268 and



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375/268 ratios in  $-\alpha^{3.7}$  heterozygotes were  $0.268 \pm 0.075$  and  $0.217 \pm 0.059$  (n=5).

The procedures described above have a number of internal controls to enhance reliability. For example, the region surrounding exon 1 of the  $\beta$ -globin locus is an excellent control for amplification efficiency since it is not the site of frequent deletions. Moreover, heretofore undetected mutations which might affect amplification efficiency would easily be detected by comparing amplicon ratios with those for controls included in each run. Deletions which might affect the control region only would result in increased (not decreased) ratios for both the  $-\alpha^{3.7}$  and --SEA/--MED amplicons when compared to control samples.

DNA controls for the --SEA and --MED deletions were not included in each run. However, the multiplex format nonetheless provided an internal control for these deletions since each --SEA or --MED deletion also affects the  $-\alpha^{3.7}$  amplicon area. Therefore, changes in the 248/268 and 375/268 ratios are in the same direction and of similar magnitude.

As shown in Table 2 (subject 13), the --BRIT deletion [see Higgs et al. (1989), supra] to the  $\alpha$  genes locus, which reduces both the 375 bp  $-\alpha^{3.7}$  and the 248 bp --SEA/--MED amplicons in much the same way as the --SEA and --MED deletions reduce these amplicon peaks, can also be detected with this M-PCR procedure. Although the primers described herein fail to distinguish between --SEA, --MED and --BRIT deletions, these conditions should be distinguishable by family history, ethnic origin, etc., if desired. It will be apparent that the teachings herein and existing knowledge of the nucleotide sequence for the  $\alpha$ -globin gene cluster permits the selection of alternative primer pairs to the primer pairs described herein, to arrive at alternative deletion-sensitive amplicons that are specific to these  $\alpha$ -globin gene deletions and other  $\alpha$ -globin gene deletions. See, e.g., Example 4.

The protocol described herein is simple enough to be performed in a routine clinical laboratory. The use of M-PCR and automated fragment analysis allows at least 30-40 samples to be processed in one day, on a single polyacrylamide gel. Although we describe quantitation from a large polyacrylamide gel on the ALF™ system, the amplicons could be quantified on any conventional fluorescence densitometer capable of scanning thin acrylamide gels. Alternatively, the amplicons are quantified by automated

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capillary electrophoresis systems (Applied Biosystems, Foster City CA). It will also be apparent that the amplicons can be quantified radiometrically (i.e., by using radiolabelled primers instead of fluorophore-labelled primers).

Since the method requires only microliter quantities of a  
5 subject's blood, screening of large populations can be performed conveniently. The skilled artisan further understands that, while template DNA was purified from human blood, DNA purified from other tissues is expected to be useful as template DNA as well.

### Example 2

#### 10 Use of M-PCR to Detect a Hypertensive Phenotype

The following exemplary data demonstrates that the M-PCR protocol described in Example 1 is useful for predicting a potential of a normotensive human subject for developing hypertension, and likewise is useful for determining a genetic abnormality that is pertinent to selection of the  
15 proper treatment of certain hypertensive human subjects.

DNA for use as M-PCR template DNA was isolated as described in Example 1 from whole blood samples of 132 African-Americans from whom blood specimens had been drawn for routine hematological testing. These patients were defined as hypertensive if they fulfilled at least  
20 one of the following criteria: (1) diastolic blood pressure exceeding 95 mm Hg, or (2) presently undergoing physician-prescribed drug therapy for previously-diagnosed hypertension.

Multiplex PCR analysis was performed as described in Example 1 with each patient's isolated DNA, using two sets of two oligonucleotide  
25 primers simultaneously in the PCR reaction mixtures: (1) Primers F37.1F and F37.3, to amplify the 375 bp  $-\alpha^{3.7}$  deletion-sensitive amplicon, and (2) Primers GH20F and PCO4, to amplify the  $\beta$ -globin control amplicon. The  $-\alpha^{3.7}$  deletion-sensitive primer pair was selected because the  $-\alpha^{3.7}$  deletion accounts for more than 95% of  $\alpha$ -thalassemia in black Americans.

30 As described in Example 1, PCR reaction products were electrophoresed on agarose gels and polyacrylamide gels, and fluorescence peak areas and peak ratios were calculated. A patient's  $\alpha$ -globin genotype was defined as "Deletion Present" (heterozygous or homozygous for an  $-\alpha^{3.7}$  deletion) if the patient's 375/268 peak ratio was determined to be in the range

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0 - 0.6; and as "Deletion Absent" if the 375/268 peak ratio was greater than 0.6. The cut-off ratio of 0.6 was selected based upon the within-run and between-run mean values for normal subjects determined in Example 1 (mean  $\pm$  SD of  $0.79 \pm 0.081$  and  $0.879 \pm 0.163$ , respectively), the ratio 0.6 being approximately two standard deviations below the expected peak ratio for normal subjects. Table 3 summarizes the data obtained from this 132 patient sample:

TABLE 3

<u>Patients</u>	<u>Deletion Present</u>	<u>Deletion Absent</u>
Normotensive	22	32
Hypertensive	55	23
Total	77	55
Hypertension Prevalence	71.4 %	41.8 %
Mean Age (Years)	56.3	56.1
Male	34 (44.2 %)	22 (40.0 %)

The data in Table 3 demonstrates a heretofore unreported and unexpected correlation between the  $-\alpha^{3.7}$  heterozygote genotype ("silent carrier" or "mild  $\alpha$ -thalassemia" genotype) and hypertension in an adult patient population. More particularly, no significant statistical differences existed in the average age or in the distribution of males and females between the 77 patients identified as possessing an  $-\alpha^{3.7}$  genotype and the 55 patients identified as normal. However, the difference in prevalence of hypertension between the two populations was statistically significant ( $p < 0.001$ ). Most of the 77 individuals identified as "deletion present" were heterozygous for the  $-\alpha^{3.7}$  deletion (e.g.,  $-\alpha^{3.7}\alpha/\alpha\alpha$ ). However, hypertension appears to correlate with homozygous individuals ( $-\alpha^{3.7}\alpha/-\alpha^{3.7}\alpha$ ) at least as strongly as with heterozygotes.

The high frequency of "Deletion Present" patients relative to the

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total number of reported patients in Table 4 (77/132) is not believed to reflect any bias in the patient population. Rather, the data reflects the fact that, for over 200 patients diagnosed as "Deletion Absent" using the M-PCR assay, the medical records of only 55 of them were examined to determine whether the patient was hypertensive or normotensive.

The foregoing data indicates persons having an  $\alpha$ -globin gene mutation, and more particularly having an  $-\alpha^{3.7}$  genotype that is common amongst black Americans, are significantly more likely to suffer from hypertension as adults than persons having a normal genotype at this locus. The data further demonstrates that the M-PCR protocol described in Example 1 is useful for determining a person's  $\alpha$ -globin genotype. Therefore, it follows that the M-PCR protocol is useful for predicting a normotensive person's potential for developing hypertension based on the person's  $\alpha$ -globin genotype, since an  $\alpha$ -globin deletion genotype correlates with an increased potential for hypertension relative to a normal genotype. Likewise, the M-PCR method is useful for determining the existence of an  $\alpha$ -globin genetic abnormality (mutation) in a hypertensive person, the presence or absence of the  $\alpha$ -globin genetic abnormality being pertinent to a physician's prescription of the proper hypertension treatment regimen.

20

### Example 3

#### Detection of $-\alpha^{4.2}$ $\alpha$ -globin gene deletions with multiplex PCR

The following procedure was performed to demonstrate the utility of the M-PCR protocol described in Example 1 for screening an individual for the presence of an  $-\alpha^{4.2}$  (leftward)  $\alpha$ -globin gene deletion. While the prevalence of this genotype is low in the general population, its prevalence can be as high as 58% in some Chinese populations.

DNA for use as M-PCR template DNA was isolated from the blood of two human subjects as described in Example 1. Using Southern hybridization analysis, the first patient's  $\alpha$ -globin genotype was confirmed to be normal, and the second patient was confirmed to be an  $-\alpha^{4.2}\alpha/\alpha\alpha$  heterozygote.

Two primers were selected and synthesized as described in Example 1 to amplify a 416 bp region that is absent in a chromosome 16 harboring an  $-\alpha^{4.2}$ , --SEA, or --MED deletion (see Fig. 1):

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Primer F42.2F (SEQ ID NO: 12): 5'-FGGGATTACAGCGTGAGCCAA-3'

Primer F42.3 (SEQ ID NO: 13): 5'-GAGGAAGGAAGGGGTGGACT-3'

Primer F42.2F is complimentary to the non-coding strand and corresponds to nucleotides 5290 to 5309 of SEQ ID NO: 1. Primer F42.3 is complimentary to nucleotides 5705 to 5686 of SEQ ID NO: 1. Primer F42.2F was fluorescein-labelled at its 5' end as described in Example 1.

M-PCR reactions were conducted as described in Example 1 using the primer pair described immediately above and the positive control primer pair described in Example 1 for amplifying a 268 bp region surrounding the 5' end of the  $\beta$ -globin gene. Reaction products were analyzed on agarose and polyacrylamide gels as previously described. The 416/268 peak area ratio was significantly reduced in the M-PCR reaction products derived from the heterozygote template DNA, confirming that the M-PCR method described herein is useful for detecting an  $-\alpha^{4.2}$  deletion.

To confirm within-run reproducibility, seven M-PCR reactions were conducted simultaneously for each individual. The 416/268 peak area ratios (+/- standard deviation) were determined to be 0.910 (+/- 0.054) for the normal subject and 0.294 (+/- 0.025) for the  $-\alpha^{4.2}$  heterozygote subject.

To confirm between-run reproducibility, five separate M-PCR reactions were conducted over a six-week period. The 416/268 peak area ratios (+/- standard deviation) were determined to be 0.945 (+/- 0.061) for the normal subject and 0.231 (+/- 0.048) for the  $-\alpha^{4.2}$  heterozygote subject.

The significant difference observed in peak ratios between normal and heterozygote individuals demonstrates that the M-PCR method described herein is useful for detecting the presence of  $-\alpha^{4.2}$  deletion mutations in a human subject's genome.

From the preceding three examples, it will be apparent that the M-PCR methods described herein can be employed to detect the presence of the major  $\alpha$ -gene deletions that afflict populations throughout the world, including the  $-\alpha^{3.7}$ ,  $-\alpha^{4.2}$ , --SEA, --MED, and other deletions.

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**Example 4****Use of M-PCR to Detect an Increased Potential  
for Developing a Blood-Related Disorder**

The following example is conducted to demonstrate that the M-PCR protocol described in the preceding examples is useful for predicting a potential of a human subject for developing a blood-related disorder, and likewise is useful for determining a genetic abnormality that is pertinent to selection of the proper treatment of certain human subjects afflicted with the blood-related disorder. Such blood-related disorders include hypertension, stroke, myocardial infarction, hemochromatosis, preeclampsia/eclampsia conditions, thalassemic disorders, diabetes, and other blood-related disorders.

DNA for use as M-PCR template DNA is isolated as described in Example 1 from whole blood samples of human subjects. It is contemplated that any purified genomic DNA from the subject is similarly suitable for use as template DNA, other tissue sources and purification methods being known in the art. In one embodiment, the human subjects are healthy human subjects who are monitored by qualified medical practitioners to determine whether the subjects subsequently develop the blood-related disorder(s) of interest. In an alternative embodiment, the human subject population includes subjects that are known to be afflicted with the blood-related disorder(s), or known to have been afflicted with the blood-related disorder(s) of interest, based upon medical examination of the subject or upon examination of the subject's medical history.

Multiplex PCR analysis is performed as described in Example 1 with each subject's isolated DNA, using two or more pairs of oligonucleotide primers simultaneously in the PCR reaction mixtures: (1) at least one pair of  $\alpha$ -globin gene deletion-sensitive primers to amplify at least one deletion-sensitive amplicon (i.e., primer pairs described in the preceding examples or primer pairs selected and synthesized according to the teachings herein; and (2) a pair of primers (e.g., Primers GH20F and PCO4) to amplify a control amplicon. For each individual pair of primers, the quantity of each primer in the M-PCR reaction mixture preferably is the same and is in the range of 0.2 to 0.4 pmol/primer. In a preferred embodiment, primer pairs for amplifying deletion-sensitive amplicons are present at about 0.4 pmol (of each primer)

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quantities, whereas about 0.2 pmol of each of the primers for amplifying the  $\beta$ -globin control amplicon are employed. In a preferred embodiment, the multiplex PCR analysis also is performed using template DNA from one or more confirmed normal ( $\alpha\alpha/\alpha\alpha$ ) subjects, to permit a determination of a control (normal subject) amplicon peak area ratio for the particular PCR reagents and reaction conditions. Similarly, multiplex PCR may be performed simultaneously using template DNA from subjects who are confirmed (e.g., by DNA sequencing or Southern hybridization analysis) to be heterozygous for a particular  $\alpha$ -gene deletion, to establish an expected amplicon peak area ratio for the particular heterozygote genotype.

PCR reaction products are electrophoresed on agarose gels and polyacrylamide gels, and fluorescence peak areas and peak area ratios are calculated. A subject's  $\alpha$ -globin genotype is defined based upon the peak area ratio of deletion-sensitive amplicons to control amplicons. Subjects are classified by their defined genotypes.

Routine statistical analysis is performed to demonstrate that the M-PCR results predict an increased potential of a human subject for developing a blood-related disorder. More particularly, the human subjects are monitored to determine whether the subjects previously have developed and/or subsequently develop the blood-related disorder(s) of interest. For any particular disorder, a greater prevalence of the disorder amongst individuals with an  $\alpha$ -globin deletion genotype versus a normal genotype suggests that individuals having the deletion genotype are more likely to develop the disorder than normal individuals. This suggestion is confirmed by routine statistical analysis, e.g., at an attained level of significance of 0.05 ( $p < 0.05$ ). Prior to performing the statistical analysis, the human subjects preferably are further classified by age, sex, and/or other characteristics that a medical practitioner familiar with the blood-related disorder of interest understands to influence an individual's likelihood of developing the disorder. In this manner, the potential confounding role of such factors in the statistical analysis is eliminated. For each  $\alpha$ -globin deletion genotype that predicts an increased potential of developing a particular blood-related disorder, subjects determined to have that particular genotype may be advised with respect to the increased potential for developing the disorder. Such genetic counseling permits a subject to make the most appropriate medical and lifestyle

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management choices prospectively.

Additional data obtained from a population of non-hospitalized individuals confirms that an increased risk exists for development of hypertension in older individuals with an  $\alpha$ -globin deletion genotype (age > 50 years: odds ratio 1.06, n=62 individuals; age > 60: odds ratio 1.21, n=33) when compared to individuals of the same age group without an  $\alpha$ -globin deletion genotype. See Armitage & Berry, *Statistical Methods in Medical Research*, 3rd Ed., London: Blackwell Scientific Publications (1994) (describing calculation of odds ratios).

Moreover, preliminary data obtained from the same group of non-hospitalized individuals demonstrated that an increased risk exists for the development of acute myocardial infarction in older individuals with an  $\alpha$ -globin deletion genotype (age > 50: odds ratio 1.07, n=62; age > 60: odds ratio 1.17, n=33) when compared to a similar control group of normal individuals. Also, an association was found to exist between an  $\alpha$ -globin deletion genotype and diabetes which is manifest in a group of 153 non-hospitalized individuals from the same geographic area as the two groups above. The odds ratio for diabetes when the  $\alpha$ -globin deletion genotype is present versus when it is absent was determined to be 2.04.

While the present invention has been described in terms of specific embodiments, it is understood that variations and modifications will occur to those skilled in the art. Accordingly, only such limitations as appear in the appended claims should be placed on the invention.



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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Bowie, Lemuel J.
- (ii) TITLE OF INVENTION: Human  $\alpha$ -Thalassemia Mutations as a Predictor of Blood-Related Disorders
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
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  - (F) ZIP: 60606-6402
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
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- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Gass, David A.
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  - (C) REFERENCE/DOCKET NUMBER: 28493/32834
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  - (C) TELEX: 25-3856

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12847 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: join(6703..6797, 6915..7119, 7262..7387)
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: join(10514..10608, 10726..10930, 11080..11205)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGATCCCCGG GGCTCTGGGC GGTGTGGGCG TAGTGAAGCC CCACGCAGCC GCCCTCCTCC      60
CCGGTCACTG ACTGGTCCTG CAGGCTCTTC ACGGTGTACC CCAGCACCAA GGTCTACTTC      120
CCGCACCTGA GCGCCTGCCA GGACGACGCA GCTGCTGAGC CACGGGAGCG CATCTGCGGC      180
TGTGGCGCGG CGGTGCAGCA CGTGGACAAC CTGCGCGCCT GAGCCCCGCTG GCGGACCTGA      240
CGCTCGTTGC GCGTGGACCC AGCCAACTTT CCGGTGAGGC CTTTCCGGCC GGGGCAATGG      300
TGCATCGCCT AGCCGGGATG GGGGGGCTCT GGGGGTCCCT AGCGGGGCAG ACCCCGTCTC      360

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ACCGGCCCCCT TCTCCTGCAG CTGCTAATCC AGTGTTTCCA CGTCGTGCTG GCCTCCCACC	420
TGCAGGACGA GTTCACCGTG CAAATGCAAG CGGCGTGGGA CAAGTTCCTG ACTGGTGTGG	480
CCGTGGTGCT GACCGAAAAA TACGCTGAGC CCTGTGCTGC GAGGCCTTGG TCTGTGCATG	540
TCAATAAACA GAGGCCCCGAA CCATCTGCCC CTGCCTGTGT GGTCTTTGGG GAGCTAGCAA	600
AGCGAGGTCA CTATTGTTGG CCAGTAAGCT CAGGGACCTA AAGGGAGCCT CTAGAACTC	660
TCAAATGCGC CCCACCCCCG GAGGTTTGTG CTCCCATGGC GAGGAGTGG ATGGGGCAGA	720
GGGAGCAGTG TGATATGGCG GGGGTAGAGA GGGTGGCCTT CGACTTCAA CCCTTGACTC	780
GGGCTTCGAA CCATACTCGT TCGCAAAGCA GTTCCCCATT CATGCATTTA TTCAGTTCAT	840
TCCTTCCCTC CATCCCCATT TCCTGCTGGC ACCTGTAGAT GCTAATCCTG GCCCTTTTTC	900
CAGAGAGATG CAGAACTGA GGTCCCAGAG CCAAATGTGC AACCTAATTC GTTGGCCAGA	960
GCAGAGGGCC GCAGACCTGT TCCTTTCCCC TTCCTTCCCC CATGGACACT TCCTCAGTGG	1020
CAAACCTGCG CTAGCCTGGT TAGCCCTCCC TGTGACCCTG CAGCCCTGGG GATGAGGTG	1080
GGAGGAAGAC CTCAGTGGCC ACAATTTGGC AGACAGAGAG GTTTAGTCTT CCAGCCTGCT	1140
CAATGACAAG CTGTGCGACC CTGGGCTGTC CCAGAGCTCT AGGCCTTTAC CTATCGAATA	1200
GAAAAACAGC GTCCAACTCA TGAGATTTTT GAAATAATTT TTGAAATCAT AACACAGGGT	1260
GGGTGCCTGC AGGGACGTTG CCACCCCACC CCTCCACCCA GCCCCAGCTG CCGTGTCTCA	1320
ATCTCTGCAG GTGCCCAGGC CAAGGCATTG CCTTCCCCAG GCTCCCTCTT CTCCCTCCCC	1380
AAGGATTGGG AAGGGAATCT TAGGGCTCCA CCCCAGGCTT TTCAGACAAA GAATAGGGGC	1440
TCAGGAAAGA TTGGGACCTT GGAGTTCTCC AATCCCTAAT AGGGTTGGGT GTGGGTTGGG	1500
CATCCTGGGT GTGTGTGGGG AGCACCTGGA CCAGGCCTGG CACCCAGGTC TGACCTGGCA	1560
GTCAGCAATG AGGTCTGAAG AGAGCTGCTG GAAGTGGAGC CCTGACTGTG AGTCGGCCAA	1620
ACTCCCCCA GCAGTCAGTG CCACAGACCT GTTGCCCTGC ACTGCCTGGG ACCCCAGCCC	1680
GGTAGTTTGG AGAACTTGGC CCCTCGTTAT CTACATCCCC CAAGTGTTTT TTTGTTTTTG	1740
GGGGTTTTTT TTTTTTTTTT TTTGCTTGT TTTTGTTTT GAGATAGGCC CTGCTCTGA	1800
CACCCCGGCT GGAGTGCAGT GGCAAGTTTT GGCTCACTGC AGCCTCAACC TCCTGGGTTC	1860
AAGCGATTCT CTGCTCTG TCTCCCGTGT AGCTGGGATT ACAGGCATGG GCCGCCATTC	1920
CTGGCTAATT TATGTATTTT TAATAGAGAC ACAGTTTCAC CATGTTGATC AGGCTGGTCT	1980
CAAACCTCTG ACCTCAAGTG ATCTGCCCTC CTGGTCTCCC AAAGTGCTGG GATGACAGGC	2040
GTGAGCCACC ACACCCAGCC CCCGCAACTG TTTACATGGA TAATTAACAA GCTTTTTGTG	2100
CCAGGCAGAG TTTGGTGTGA AAGCAGCTTA TGTTTCACTT TGGAAAACT GTGCTCTTCT	2160
CCCCATCCAG GAAGCTGCCT GGGTCTGGGC CATATGTGGA TACCTTATGG GTATAAGCTG	2220
CTCAGGACCC TGTGTGGAAG CTCAGGACAA TGCCAGCGGG AAGGCTACCA TGTGGAGAGC	2280
TGTCTCTGTT TGGGCAGGAC TAAGAGACGC AGGGAACCTT GGGAACCTGT CTACTCTCAC	2340
TCACTCCTCC TCCCCTTCC TTCCAGGCAC CTCTGCAACT TGCCAGCCAA TGACCCTGCA	2400
TCCCAGGCAT AAGAGCTCCT ACTCTCCCC ACCTTTCCT TTTGAGCTTA CACAGACTCA	2460
GAAATTAAGC TGCCGTGGTG CTGTCTCTG AGGACAAGGC TAACACCAAG GCGGTCTGGG	2520

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AGAAAGTTGG	CGACCACACT	GCTGGCTATG	CCACGGAGGC	CCTGGAGAGG	CAAGAACCCT	2580
CCTCTCCCTG	CTCACACCTT	GGGTCCAACG	CCCCTCCAG	GGCTCCACTG	GCCACCCCTA	2640
ACTATTCTTA	CCCTGGACCC	AGCCCCCAGC	CCCTCACTCT	TTGCTTCCCC	CTGAAGCATG	2700
TTCTTGACCT	TCCTCTCACT	TGGCCCTGAG	TTATGGCTCA	GCCCAGATCA	AGAAACAATG	2760
CAAGTAGGTG	GCCGACACGC	TGACCAATGC	CGTGGTCCAC	TTAGATGACA	TGCCCCAATGA	2820
TGTGTCTGAG	GTGAGGAAGC	TGCATGTCCA	CGAGCTGTGG	GTGGACCCAG	GCAACATCAG	2880
GGAGAGCTTT	GGGCTGGGAG	GAATCTAGGG	TGTGGGGGCA	GCTGGCCTTC	CTCATAGGAC	2940
AGACCCTCCC	ACGCGTTCAG	GGAGGTGGAG	CACAGGTGGC	AGTAGTATCT	GCATCCCCTG	3000
ACTCTCTCTC	CACAGTTCTT	GGCTAAATGC	CTGCTGGTGA	CCTAGGCTTG	GCACAGCCTT	3060
CCCGGTTTAC	CCATGTGGTG	CCTCCATGGA	CAAATTATTT	GCTTTTGTGA	GTGCTGTGTT	3120
GACCTAAAAA	CACCATTAAAG	CTAGAGCATT	GGTGGTCATG	CCCCCTGCCT	GCTGGGCCTC	3180
CCACCAGGCC	CGCCTCCCCT	CCCTGCCCCA	GCACTTCCTG	ATCTTTGAAT	GAAGTCCGAG	3240
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TGTTTACCTG	TCTCAGACCA	AGGACCTCTC	TGCAGCTGCA	TGGGGCTGGG	GAGGGAGAAC	3360
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TGCCAGAGGG	TAGGTGGCTG	GAGTGAGGGT	GCTGGCCCTA	CTCACACTTC	CTGTGTCAATG	3720
GTGACCCTCT	GAGAGCAGCC	CAGTCAGTGG	GGAAGGAGGA	AGGGGCTGGG	ATGCTCACAG	3780
CCGGCAGCCC	ACACCTAGGG	AGACTCTTCA	GCAGAGCACC	TTGCGGCCTT	ACTCCTGCAC	3840
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GCTAATTGCC	CCACCCAGGG	CCTCTGGGAC	CTCCTGGTGC	TTCTGCTTCC	TGTGCTGCCA	3960
GCAACTTCTG	GAAACGTCCC	TGTCCCCGGT	GCTGAAGTCC	TGGAATCCAT	GCTGGGAAGT	4020
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CCTGCCCCAC	AGCAAGCCTC	CCCCTCCACA	CTCACAGTAC	TGAATTGAGC	TTTGGGTAGG	4140
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GTTCCCTCCC	CCTCTCCACC	ACCCACTCTT	CCTGTGCCTC	ACAGCCCAGA	GCATTGTTAT	4260
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CAGCCTGACC	AATATGGAGA	AACCCAGTT	ATACTAAAAA	TACAAAATTA	GCTGGGTGTG	4440
GTGGCGCATG	CCTGTAATCC	TAGCTACTAG	GAAGGCTGAG	GCAGGAGAAT	CGCTTGAACC	4500
CGGGAGGTGG	AGGTTGAGGT	GAGCCGAGAT	CACGCCATTG	CACTCCAGCC	TGGGCAACAA	4560
GAGCAAACT	CCGTCTCAAA	AAATAAATAA	ATAAATAAAT	AAATAAACTA	AAATCTATCC	4620
ATGCTTTCAC	ACACACACAC	ACACACACAC	ACACACACCT	TTTTTGTGTT	ACTAAAGTAG	4680

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GAGAGTGTCT	CTCTTTCCTG	TCTCCTCACA	CCCCCCCCCA	GAAGAGACCA	AAATGAAGGG	4740
TTTGGAAGTC	ACGCCATGGG	CCCCATCCCA	TGCTGAGGGA	ACACAGCTAC	ATCTACAAC	4800
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GCTCGCCCAT	TCCACAGTCT	CACTGATTAT	TACTATGTTT	CCATCATGAT	CCCCCAAAA	4980
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TCCACCTCGC	AGGTTACGCG	CATTCTCCTC	CCTCAGCCTC	CCGAGTCGCT	GAGTAGCTGG	5160
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CACTGCATTA	GCGAGGATGG	TCTCGATCTC	CTGACCTCGC	ATCTGCCAGC	CTCAGCCTTC	5280
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GTGGGAGAGT	CAGTCACACA	AACTGTGAGT	CCATGACTTG	GGGCTTAGCC	AGTACCCACC	5940
ACCCACGCG	CCACCCACCA	ACCCCGGGTA	GAGGAGTCTG	AATCTGGAGC	CGCCCCCAGC	6000
CCAGCCCCGT	GCTTTTTCG	TCCTGGTGT	TGTTCTTTC	CGGTGCCTGT	CACTCAAGCA	6060
CACTAGTGAC	TATCGCCAGA	GGGAAAGGGA	GCTGCAGGAA	GCGAGGCTGG	AGAGCAGGAG	6120
GGGCTCTGCG	CAGAAATTCT	TTTGAGTTCC	TATGGGCCAG	GGCGTCCGGG	TGCGCGCATT	6180
CCTCTCCGCC	CCAGGATTGG	GCGAAGCCCT	CCGGCTCGCA	CTCGCTCGCC	CGTGTGTTCC	6240
CCGATCCCGC	TGGAGTCGAT	GCGCTCCAG	CGCGTGCCAG	GCCGGGGCGG	GGGTGCGGGC	6300
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CGGGGTGCAC	GAGCCGACAG	CGCCCGACCC	CAACGGGCGG	GCCCCGCCAG	CGCCGCTACC	6420
GCCCTGCCCC	GGCGAGCGGG	ATGGGCGGGA	GTGGAGTGGC	GGGTGGAGGG	TGGAGACGTC	6480
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AGCGCCGCCC	GGCCGGGCGT	GCCCCGCGC	CCCAAGCATA	AACCCTGGCG	CGCTCGCGGC	6660
CCGGCACTCT	TCTGGTCCCC	ACAGACTCAG	AGAGAACCCA	CC ATG GTG CTG TCT		6714
				Met Val Leu Ser		

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CCT GCC GAC AAG ACC AAC GTC AAG GCC GCC TGG GGT AAG GTC GGC GCG Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala 5 10 15 20	6762
CAC GCT GGC GAG TAT GGT GCG GAG GCC CTG GAG AG GTGAGGCTCC His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg 25 30	6807
CTCCCCCTGCT CCGACCCGGG CTCTCGCCC GCCCGGACCC ACAGGCCACC CTCAACCGTC	6867
CTGGCCCCGG ACCCAAACCC CACCCCTCAC TCTGCTTCTC CCCGCAG G ATG TTC Met Phe	6921
CTG TCC TTC CCC ACC ACC AAG ACC TAC TTC CCG CAC TTC GAC CTG AGC Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp Leu Ser 35 40 45 50	6969
CAC GGC TCT GCC CAG GTT AAG GGC CAC GGC AAG AAG GTG GCC GAC GCC His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala Asp Ala 55 60 65	7017
CTG ACC AAC GCC GTG GCG CAC GTG GAC GAC ATG CCC AAC GCG CTG TCC Leu Thr Asn Ala Val Ala His Val Asp Met Pro Asn Ala Leu Ser 70 75 80	7065
GCC CTG AGC GAC CTG CAC GCG CAC AAG CTT CGG GTG GAC CCG GTC AAC Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro Val Asn 85 90 95	7113
TTC AAG GTGAGCGCG GGCCGGGAGC GATCTGGGTC GAGGGGCGAG ATGGCGCCTT Phe Lys 100	7169
CCTCTCAGGG CAGAGGATCA CGCGGGTTGC GGGAGGTGTA GCGCAGGCGG CGGCTGCGGG	7229
CCTGGGCCCGC ACTGACCCTC TTCTCTGCAC AG CTC CTA AGC CAC TGC CTG CTG Leu Leu Ser His Cys Leu Leu 105	7282
GTG ACC CTG GCC GCC CAC CTC CCC GCC GAG TTC ACC CCT GCG GTG CAC Val Thr Leu Ala Ala His Leu Pro Ala Glu Phe Thr Pro Ala Val His 110 115 120	7330
GCC TCC CTG GAC AAG TTC CTG GCT TCT GTG AGC ACC GTG CTG ACC TCC Ala Ser Leu Asp Lys Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser 125 130 135	7378
AAA TAC CGT TAAGCTGGAG CCTCGGTAGC CGTTCCTCCT GCCCGATGGG Lys Tyr Arg 140	7427
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TGAGTGGGCG GCAGCCTGTG TGTGCTGGG TTCTCTCTGT CCCGGAATGT GCCAACAATG	7547
GAGGTGTTTA CCTGTCTCAG ACCAAGGACC TCTCTGCAGC TGCATGGGGC TGGGGAGGGA	7607
GAAGTCAGG GAGTATGGGA GGGGAAGCTG AGGTGGGCCT GCTCAAGAGA AGGTGCTGAA	7667
CCATCCCCTG TCCTGAGAGG TGCCAGGCCT GCAGGCAGTG GCTCAGAAGC TGGGGAGGAG	7727
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CTGGTTGAAC CCAGTTAACA TACGCTCTCC ATCAAAACAA AACGAAACAA AACAACTAG	7847
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TTCTGCCCAG AGGGTAGGTG GCTGGAGTGA GGGTGCTGGC CCTACTCACA CTTCCTGTGT	7967
CACGGTGACC CTCTGAGAGC AGCCAGTCA GTGGGGAAGG AGGAAGGGGC TGGGATGCTC	8027

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ACAGCCGGCA	GCCACACCT	AGGGAGACTC	TTCAGCAGAG	CACCTTGCGG	CCTTACTCCT	8087
GCACGTCTCC	TGCAGTTTGT	AAGGTGCATT	CAGAACTCAC	TGTGTGCCCC	GCCCTGAGCT	8147
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AAGTTGCACA	GCCCATCTGG	CTCTCAGCCA	GCCTAGGAAC	ATGAGCAGCA	CTTCCAACCC	8327
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GAGGGTGGAG	AGGACCCTGT	CACCGCTTTC	CTTCTGGACA	TGGACCTCTC	TGAATTGTTG	8447
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TTATTTCAGC	AGGAAACACTT	TAAAAATTA	ACTAAATCC	GACAGGCACC	GTGGCTCACC	8567
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CATCCCCCCA	AAAAACATCA	CTTTTCATGC	ACAGGGATGC	ACCCACTGGC	ACTCCTGCAC	9227
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CCTTTCCCCT	CCCCGAGCCA	AGCCTCCTCC	CTCCTCCACC	TCCTCCACCT	AATACATATC	9407
CTTAAGTCTC	ACCTCCTCCA	GGAAGCCCTC	AGACTAACCC	TGGTCCCCTT	GAATGCCTCG	9467
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TATTGCTTTG	TTTACCTGTT	TAACCAGTAT	TTACCTAGCA	AGTCTTCCAT	CAGATAGCAT	9647
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CACAACTGT	GAGTCCATGA	CTTGGGGCTT	AGCCAGCACC	CACCACCCCA	CGCGCCACCC	9767
CACAACCCCG	GGTAGAGGAG	TCTGAATCTG	GAGCCGCCCC	CAGCCCAGCC	CCGTGCTTTT	9827
TGCGTCCTGG	TGTTTATTCC	TTCCCGGTGC	CTGTCACTCA	AGCACACTAG	TGACTATCGC	9887
CAGAGGGA	GGGAGCTGCA	GGAAGCGAGG	CTGGAGAGCA	GGAGGGGCTC	TGCGCAGAAA	9947
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CGATGCGCGT	CCAGCGCGTG	CCAGGCCGGG	GCGGGGGTGC	GGGCTGACTT	TCTCCCTCGC	10127
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CCCCACAGAC	TCAGAGAGAA	CCCACC	ATG GTG CTG TCT CCT GCC GAC AAG ACC			10540
			Met Val Leu Ser Pro Ala Asp Lys Thr			
			1	5		
AAC GTC AAG GCC GCC TGG GGT AAG GTC GGC GCG CAC GGT GGC GAG TAT						10588
Asn Val Lys Ala Ala Trp Gly Lys Val Gly Ala His Ala Gly Glu Tyr						
10		15		20	25	
GGT GCG GAG GCC CTG GAG AG	GTGAGGCTCC	CTCCCCTGCT	CCGACCCGGG			10638
Gly Ala Glu Ala Leu Glu Arg						
			30			
CTCCTCGCCC	GCCCGGACCC	ACAGGCCACC	CTCAACCGTC	CTGGCCCCGG	ACCCAAACCC	10698
CACCCCTCAC	TCTGCTTCTC	CCCGCAG G	ATG TTC CTG TCC TTC CCC ACC ACC			10750
			Met Phe Leu Ser Phe Pro Thr Thr			
				35	40	
AAG ACC TAC TTC CCG CAC TTC GAC CTG AGC CAC GGC TCT GCC CAG GTT						10798
Lys Thr Tyr Phe	Pro His Phe Asp Leu Ser His Gly Ser Ala Gln Val					
	45		50		55	
AAG GGC CAC GGC AAG AAG GTG GCC GAC GCC CTG ACC AAC GCC GTG GCG						10846
Lys Gly His Gly Lys Lys Val Ala Asp Ala Leu Thr Asn Ala Val Ala						
	60		65		70	
CAC GTG GAC GAC ATG CCC AAC GCG CTG TCC GCC CTG AGC GAC CTG CAC						10894
His Val Asp Asp Met Pro Asn Ala Leu Ser Ala Leu Ser Asp Leu His						
	75		80		85	
GCG CAC AAG CTT CGG GTG GAC CCG GTC AAC TTC AAG GTGAGCGGCG						10940
Ala His Lys Leu Arg Val Asp Pro Val Asn Phe Lys						
	90		95		100	
GGCCGGGAGC	GATCTGGGTC	GAGGGGCGAG	ATGGCGCCTT	CCTCGCAGGG	CAGAGGATCA	11000
CGCGGGTTGC	GGGAGGTGTA	GCGCAGGCGG	CGGCTGCGGG	CCTGGGCCCT	CGGCCCCACT	11060
GACCTCTTTC	TCTGCACAG	CTC CTA AGC CAC TGC CTG CTG GTG ACC CTG GCC				11112
		Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala				
			105		110	
GCC CAC CTC CCC GCC GAG TTC ACC CCT GCG GTG CAC GCC TCC CTG GAC						11160
Ala His Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp						
	115		120		125	
AAG TTC CTG GCT TCT GTG AGC ACC GTG CTG ACC TCC AAA TAC CGT						11205
Lys Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg						
	130		135		140	
TAAGCTGGAG	CCTCGGTGGC	CATGCTTCTT	GGCCCTTGGG	CCTCCCCCCA	GCCCTCCTC	11265
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GTGTGCCTGA	GTTTTTTTCCC	TCAGAAACGT	GCCAGCATGG	GCGTGGACAG	CAGCTGGGAC	11385
ACACATGGCT	AGAACCTCTC	TGCAGCTGGA	TAGGGTAGGA	AAAGGCAGGG	GCGGGAGGAG	11445
GGGATGGAGG	AGGGAAAGTG	GAGCCACCGC	GAAGTCCAGC	TGGAAAAACG	CTGGACCCTA	11505
GAGTGCTTTG	AGGATGCATT	TGCTCTTTTC	CGAGTTTTAT	TCCAGACTT	TTCAGATTCA	11565

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ATGCAGGTTT GCTGAAATAA TGAATTTATC CATCTTTACG TTTCTGGGCA CTCTTG TGCC 11625
AAGAACTGGC TGGCTTTCTG CCTGGGACGT CACTGGTTTC CCAGAGGTCC TCCCACATAT 11685
GGGTGGTGGG TAGGTCAGAG AAGTCCCACT CCAGCATGGC TGCATTGATC CCCCATCGTT 11745
CCCACTAGTC TCCGTAAAAC CTCCCAGATA CAGGCACAGT CTAGATGAAA TCAGGGGTGC 11805
GGGGTGCAAC TGCAGGCCCC AGGCAATTCA ATAGGGGGCTC TACTTTTACC CCCAGGTCAC 11865
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GAGGCGGAGG TTGTAGTGAG TCAAGATCGC GCCATCGCAC TCCAGCTTGG GCAACAAGAG 12705
CGAAACTCTG TCTCAAAAAA AAATTTAATC TAATTTAATT TAATTTAAAA ATTAGCACGG 12765
TGTTTGGGCA CAGTGGCCTC ACGCCTGTAA TCCCAGCACT TTGGGAAGCC AAGGTGGGCA 12825
GATCACAAGG TCAGGGGAAT TC 12847

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## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 142 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Val Leu Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly
 1             5             10             15
Lys Val Gly Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg
          20             25             30
Met Phe Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp
          35             40             45
Leu Ser His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala
          50             55             60
Asp Ala Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala
          65             70             75             80
Leu Ser Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro
          85             90             95

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Val Asn Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala  
                   100                  105                  110  
 His Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys  
                   115                  120                  125  
 Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg  
                   130                  135                  140

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 142 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Val Leu Ser Pro Ala Asp Lys Thr Asn Val Lys Ala Ala Trp Gly  
   1                  5                  10                  15  
 Lys Val Gly Ala His Ala Gly Glu Tyr Gly Ala Glu Ala Leu Glu Arg  
                   20                  25                  30  
 Met Phe Leu Ser Phe Pro Thr Thr Lys Thr Tyr Phe Pro His Phe Asp  
                   35                  40                  45  
 Leu Ser His Gly Ser Ala Gln Val Lys Gly His Gly Lys Lys Val Ala  
                   50                  55                  60  
 Asp Ala Leu Thr Asn Ala Val Ala His Val Asp Asp Met Pro Asn Ala  
                   65                  70                  75                  80  
 Leu Ser Ala Leu Ser Asp Leu His Ala His Lys Leu Arg Val Asp Pro  
                   85                  90                  95  
 Val Asn Phe Lys Leu Leu Ser His Cys Leu Leu Val Thr Leu Ala Ala  
                   100                  105                  110  
 His Leu Pro Ala Glu Phe Thr Pro Ala Val His Ala Ser Leu Asp Lys  
                   115                  120                  125  
 Phe Leu Ala Ser Val Ser Thr Val Leu Thr Ser Lys Tyr Arg  
                   130                  135                  140

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACCATCCCC CAAAAACAT

20

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 20 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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CTCTAACCAT CACACAAGTA

20

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACTGGAGAGG AGAGCGGGGC

20

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGGCTGCGGG AAGGACATCA

20

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAAGAGCCAA GGACAGGTAC

20

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAACTTCATC CAGTTCACC

20

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2500 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(687..778, 909..1131, 1982..2107)

- 49 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTAGACATA ATTTATTAGC ATGCATGAGC AAATTAAGAA AAACAACAAC AAATGAATGC	60
ATATATATGT ATATGTATGT GTGTATATAT ACACATATAT ATATATATTT TTTTCTTTT	120
CTTACCAGAA GGTTTTAATC CAAATAAGGA GAAGATATGC TTAGAACTGA GGTAGAGTTT	180
TCATCCATTC TGTCCTGTAA GTATTTTGCA TATTCTGGAG ACGCAGGAAG AGATCCATCT	240
ACATATCCCA AAGCTGAATT ATGGTAGACA AAGCTCTTCC ACTTTTAGTG CATCAATTTT	300
TTATTTGTGT AATAAGAAAA TTGGGAAAC GATCTTCAAT ATGCTTACCA AGCTGTGATT	360
CCAAATATTA CGTAAATACA CTTGCAAAGG AGGATGTTTT TAGTAGCAAT TTGTACTGAT	420
CCCTATCCGCC CAAGAGATAT ATCTTAGAGG GAGGGCTGAG GGTGTGAGT CCAACTCTTA	480
AGCCAGTGCC AGAAGAGCCA AGGACAGGTA CGGCTGTCAT CACTTAGACC TCACCCTGTG	540
GAGCCACACC CTAGGGTTGG CCAATCTACT CCCAGGAGCA GGGAGGGCAG GAGCCAGGGC	600
TGGGCATAAA AGTCAGGGCA GAGCCATCTA TTGCTTACAT TTGCTTCTGA CACAACTGTG	660
TTCACTAGCA ACCTCAAACA GACACC ATG GTG CAC CTG ACT CCT GAG GAG AAG	713
Met Val His Leu Thr Pro Glu Glu Lys	
1 5	
TCT GCC GTT ACT GCC CTG TGG GGC AAG GTG AAC GTG GAT GAA GTT GGT	761
Ser Ala Val Thr Ala Leu Trp Gly Lys Val Asn Val Asp Glu Val Gly	
10 15 20 25	
GGT GAG GCC CTG GGC AG GTTGGTATCA AGGTTACAAG ACAGGTTTAA	808
Gly Glu Ala Leu Gly Arg	
30	
GGAGACCAAT AGAAACTGGG CATGTGGAGA CAGAGAAGAC TCTTGGGTTT CTGATAGGCA	868
CTGACTCTCT CTGCCTATTG GTCTATTTTC CCACCCTTAG G CTG CTG GTG GTC	921
Leu Leu Val Val	
35	
TAC CCT TGG ACC CAG AGG TTC TTT GAG TCC TTT GGG GAT CTG TCC ACT	969
Tyr Pro Trp Thr Gln Arg Phe Phe Glu Ser Phe Gly Asp Leu Ser Thr	
40 45 50	
CCT GAT GCT GTT ATG GGC AAC CCT AAG GTG AAG GCT CAT GGC AAG AAA	1017
Pro Asp Ala Val Met Gly Asn Pro Lys Val Lys Ala His Gly Lys Lys	
55 60 65	
GTG CTC GGT GCC TTT AGT GAT GGC CTG GCT CAC CTG GAC AAC CTC AAG	1065
Val Leu Gly Ala Phe Ser Asp Gly Leu Ala His Leu Asp Asn Leu Lys	
70 75 80	
GGC ACC TTT GCC ACA CTG AGT GAG CTG CAC TGT GAC AAG CTG CAC GTG	1113
Gly Thr Phe Ala Thr Leu Ser Glu Leu His Cys Asp Lys Leu His Val	
85 90 95	
GAT CCT GAG AAC TTC AGG GTGAGTCTAT GGGACCCTTG ATGTTTTCTT	1161
Asp Pro Glu Asn Phe Arg	
100 105	
TCCCTTCTT TTCTATGGTT AAGTTCATGT CATAGGAAGG GGAGAAGTAA CAGGGTACAG	1221
TTTAGAATGG GAAACAGACG AATGATTGCA TCAGTGTGGA AGTCTCAGGA TCGTTTTAGT	1281
TTCTTTTATT TGCTGTTTAT AACAATTGTT TTCTTTTGTG TAATTCTTGC TTTCTTTTTT	1341
TTTCTTCTCC GCAATTTTAA CTATTATACT TAATGCCTTA ACATTGTGTA TAACAAAAGG	1401
AAATATCTCT GAGATACATT AAGTAACTTA AAAAAAACT TTACACAGTC TGCCTAGTAC	1461

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ATTACTATTT GGAATATATG TGTGCTTATT TGCATATTCA TAATCTCCCT ACTTTATTTT 1521  
 CTTTATTTT TAATTGATAC ATAATCATT TACATATTTA TGGGTAAAG TGTAATGTTT 1581  
 TAATATGTGT ACACATATTG ACCAAATCAG GGTAAATTTG CATTTGTAAT TTTAAAAAAT 1641  
 GCTTCTTCT TTTAATATAC TTTTTGTTT ATCTTATTTT TAATACTTTC CCTAATCTCT 1701  
 TTCTTTCAGG GCAATAATGA TACAATGTAT CATGCCTCTT TGCACCATT CAAAGAATAA 1761  
 CAGTGATAAT TTCTGGGTTA AGGCAATAGC AATATTTCTG CATATAAATA TTTCTGCATA 1821  
 TAAATTGTAA CTGATGTAAG AGGTTTCATA TTGCTAATAG CAGCTACAAT CCAGCTACCA 1881  
 TTCTGCTTTT ATTTTATGGT TGGGATAAGG CTGGATTATT CTGAGTCCAA GCTAGGCCCT 1941  
 TTGCTAATC ATCTTCATAC CTCTTATCTT CCTCCACAC CTC CTG GGC AAC GTG 1996  
 Leu Leu Gly Asn Val 110  
 CTG GTC TGT GTG CTG GCC CAT CAC TTT GGC AAA GAA TTC ACC CCA CCA 2044  
 Leu Val Cys Val Leu Ala His His Phe Gly Lys Glu Phe Thr Pro Pro 115 120 125  
 GTG CAG GCT GCC TAT CAG AAA GTG GTG GCT GGT GTG GCT AAT GCC CTG 2092  
 Val Gln Ala Ala Tyr Gln Lys Val Val Ala Gly Val Ala Asn Ala Leu 130 135 140  
 GCC CAC AAG TAT CAC TAAGCTCGCT TTCTTGCTGT CCAATTTCTA TTAAAGGTTT 2147  
 Ala His Lys Tyr His 145  
 CTTTGTTCCT TAAGTCCAAC TACTAACTG GGGGATATTA TGAAGGGCCT TGAGCATCTG 2207  
 GATTCTGCCT AATAAAAAAC ATTTATTTTC ATTGCAATGA TGTATTTAAA TTATTTCTGA 2267  
 ATATTTTACT AAAAAGGGAA TGTGGGAGGT CAGTGCATT AAAACATAAA GAAATGAAGA 2327  
 GCTAGTTCAA ACCTTGGGAA AATACACTAT ATCTTAACT CCATGAAAGA AGGTGAGGCT 2387  
 GCAAACAGCT AATGCACATT GGCAACAGCC CTGATGCCTA TGCCTTATTC ATCCCTCAGA 2447  
 AAAGGATTCA AGTAGAGGCT TGATTTGGAG GTTAAAGTTT TGCTATGCTG TAT 2500

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 147 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Val His Leu Thr Pro Glu Glu Lys Ser Ala Val Thr Ala Leu Trp  
 1 5 10 15  
 Gly Lys Val Asn Val Asp Glu Val Gly Gly Glu Ala Leu Gly Arg Leu  
 20 25 30  
 Leu Val Val Tyr Pro Trp Thr Gln Arg Phe Phe Glu Ser Phe Gly Asp  
 35 40 45  
 Leu Ser Thr Pro Asp Ala Val Met Gly Asn Pro Lys Val Lys Ala His  
 50 55 60  
 Gly Lys Lys Val Leu Gly Ala Phe Ser Asp Gly Leu Ala His Leu Asp  
 65 70 75 80  
 Asn Leu Lys Gly Thr Phe Ala Thr Leu Ser Glu Leu His Cys Asp Lys  
 85 90 95

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Leu His Val Asp Pro Glu Asn Phe Arg Leu Leu Gly Asn Val Leu Val  
                   100                                  105                                  110  
 Cys Val Leu Ala His His Phe Gly Lys Glu Phe Thr Pro Pro Val Gln  
                   115                                  120                                  125  
 Ala Ala Tyr Gln Lys Val Val Ala Gly Val Ala Asn Ala Leu Ala His  
                   130                                  135                                  140  
 Lys Tyr His  
 145

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGATTACAG CGTGAGCCAA

20

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGGAAGGAA GGGGTGGACT

20

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What is claimed is:

1. A method of screening a human subject for a risk of developing a blood-related disorder comprising the steps of:
  - (a) assaying genomic DNA of a human subject to determine a presence  
5 or an absence of an  $\alpha$ -globin mutation; and
  - (b) correlating the presence of an  $\alpha$ -globin mutation in the genomic DNA to a risk of developing a blood-related disorder, wherein said blood-related disorder is selected from the group consisting of hypertension, stroke, myocardial infarction, diabetes, hemochromatosis, a preeclampsia condition,  
10 and eclampsia.
2. The method of claim 1 wherein the human subject is normotensive and the blood-related disorder is hypertension.
3. The method of claim 2 wherein the genomic DNA is assayed to determine the presence or the absence of an  $\alpha$ -globin deletion mutation.
- 15 4. The method of claim 3 wherein the  $\alpha$ -globin deletion mutation is selected from the group consisting of an  $-\alpha^{3.7}$  deletion, an --MED deletion, an --SEA deletion, an  $-\alpha^{4.2}$  deletion, and an --BRIT deletion.
5. The method of claim 3 wherein the genomic DNA is assayed to determine the presence or the absence of an  $-\alpha^{3.7}$  deletion mutation.
- 20 6. The method of claim 5 wherein the subject is heterozygous for an  $-\alpha^{3.7}$  deletion.

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7. A method of determining an increased potential for developing hypertension in a normotensive human individual comprising the steps of:

- (a) isolating DNA from said human individual;
- 5 (b) assaying said DNA for the presence of a deletion relative to DNA of a normal human subject, said deletion comprising a reduction in the number of  $\alpha$ -globin genes in the genome of the normotensive human individual; and
- (c) determining a potential for developing hypertension in said normotensive human individual, wherein the presence of said deletion in the
- 10 DNA isolated from the normotensive human individual is indicative of an increased potential for developing hypertension.

8. The method of claim 7 wherein the deletion is present in the DNA of the normotensive human individual.

9. The method of claim 7 wherein in step (b) said deletion is a

15 deletion of at least about 3.7 kilobases of DNA.

10. The method of claim 7 wherein the deletion is selected from the group consisting of an  $-\alpha^{3.7}$  deletion, an --MED deletion, an --SEA deletion, an  $-\alpha^{4.2}$  deletion, and an --BRIT deletion.

11. The method of claim 7 wherein the deletion is an  $-\alpha^{3.7}$

20 deletion.

12. The method of claim 7 wherein step (b) comprises at least one of:

- (i) determining a partial nucleotide sequence of the subject's DNA, said partial nucleotide sequence indicating the presence or absence of said deletion;
- 25 (ii) performing a restriction endonuclease digestion of the subject's DNA and a Southern hybridization, said Southern hybridization indicating the presence or absence of said deletion; and
- (iii) performing a multiplex polymerase chain reaction to determine the presence or absence of said deletion.

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13. The method of claim 7 wherein step (b) comprises performing a multiplex polymerase chain reaction assay for the presence of said deletion.

14. A method for determining an increased potential for  
5 developing hypertension in a human individual comprising the steps of:  
    (a) isolating genomic DNA from said human individual;  
    (b) performing a multiplex polymerase chain reaction with said genomic  
DNA to amplify a marker portion and a control portion thereof, wherein the  
presence of an  $\alpha$ -globin gene deletion in said genomic DNA reduces the  
10 quantity of marker amplicons produced in the multiplex polymerase chain  
reaction relative to the quantity of control amplicons produced in said  
multiplex polymerase chain reaction; and  
    (c) determining a potential for developing hypertension from the  
quantity of marker amplicons relative to the quantity of control amplicons.

15. The method of claim 14 wherein step (c) further comprises  
the steps of:  
    (i) determining a ratio of marker amplicons to control amplicons  
produced in the multiplex polymerase chain reaction; and  
    (ii) comparing the ratio of step (i) to a ratio of marker amplicons to  
20 control amplicons produced in a multiplex polymerase chain reaction  
performed with genomic DNA of a normal human subject, wherein a lower  
marker amplicon to control amplicon ratio of step (i) indicates an increased  
potential for developing hypertension.

16. The method of claim 15 wherein step (b) further comprises  
25 the step of preparing a polymerase chain reaction solution, said solution  
comprising genomic DNA isolated from said human individual, a first pair of  
oligonucleotide primers for amplifying said marker portion of said DNA, and a  
second pair of oligonucleotide primers for amplifying said control portion of  
said DNA.



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17. The method of claim 16 wherein said first pair of primers consists of a primer having the nucleotide sequence of SEQ ID NO: 4 and a primer having the nucleotide sequence of SEQ ID NO: 5.

18. The method of claim 17 wherein said second pair of  
5 primers consists of a primer having the nucleotide sequence of SEQ ID NO: 8 and a primer having the nucleotide sequence of SEQ ID NO: 9.

19. The method of claim 18 wherein in step (ii) said lower ratio is less than or equal to 0.6.

20. The method of claim 15 further comprising:  
10 isolating genomic DNA from a normal human being;  
performing a control multiplex polymerase chain reaction with the genomic DNA of the normal human being to amplify a marker portion and a control portion thereof; and  
determining a ratio of marker amplicons to control amplicons produced  
15 in the control multiplex polymerase chain reaction;  
wherein in step (ii), a lower marker amplicon to control amplicon ratio for the human individual compared to the normal human being indicates an increased potential for developing hypertension.

21. A genetic counseling method comprising the steps of:  
20 (a) isolating genomic DNA from a human individual;  
(b) assaying said DNA for a deletion relative to DNA of a normal human subject, said deletion reducing the number of  $\alpha$ -globin genes in the genome of the human individual;  
(c) determining a potential for developing a blood-related disorder in  
25 said human individual, wherein the presence of said deletion in the genomic DNA of the human individual is indicative of an increased potential for developing said blood-related disorder, said blood-related disorder selected from the group consisting of hypertension, stroke, myocardial infarction, diabetes, hemochromatosis, a preeclampsia condition, and eclampsia; and  
30 (d) advising the human individual with respect to the individual's potential for developing said blood-related disorder.

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22. A kit for screening a human subject for an increased potential of developing a blood-related disorder, said kit comprising:

an assay means for assaying genomic DNA from a human subject for the presence of an  $\alpha$ -globin mutation, wherein said assay means produces a  
5 positive  $\alpha$ -globin assay result if said  $\alpha$ -globin mutation is present in the DNA of said subject, and said assay means produces a negative assay result if the DNA of said subject is free of said  $\alpha$ -globin mutation; and

a means for correlating a positive  $\alpha$ -globin assay result to an increased potential of developing a blood-related disorder, said disorder selected from  
10 the group consisting of hypertension, stroke, myocardial infarction, diabetes, hemochromatosis, a preeclampsia condition, and eclampsia.

23. The kit of claim 22 wherein the assay means comprises a pair of  $\alpha$ -globin deletion-sensitive oligonucleotide primers.

24. The kit of claim 23 wherein the assay means further  
15 comprises first and second samples of human genomic DNA, said first sample having a homozygous normal  $\alpha$ -globin genotype, said second sample having a heterozygous  $\alpha\alpha/\alpha$ -globin-deletion genotype.

25. The kit of claim 24 wherein the second sample of human genomic DNA has a heterozygous  $\alpha\alpha/-\alpha^{3.7}\alpha$  genotype.

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26. A method for assaying for a mild  $\alpha$ -thalassemia genotype in a human individual comprising the steps of:

- (a) isolating genomic DNA from said human individual;
- (b) performing a multiplex polymerase chain reaction with said genomic DNA to amplify a marker portion and a control portion thereof, wherein the presence of an  $\alpha$ -globin gene deletion in said genomic DNA reduces the quantity of marker amplicons produced in the multiplex polymerase chain reaction relative to the quantity of control amplicons produced in said multiplex polymerase chain reaction;
- (c) determining a ratio of marker amplicons to control amplicons produced in the multiplex polymerase chain reaction; and
- (d) comparing the ratio of step (c) to a ratio of marker amplicons to control amplicons produced in a multiplex polymerase chain reaction performed with genomic DNA of a normal human subject, wherein a mild  $\alpha$ -thalassemia genotype in said individual correlates with a lower marker amplicon to control amplicon ratio of step (c) compared to the ratio of marker amplicons to control amplicons produced in a multiplex polymerase chain reaction performed with genomic DNA of a normal human subject.

27. The method of claim 26 for assaying for the presence of a -  $\alpha^{3.7}\alpha/\alpha\alpha$  genotype in a human individual.

28. The method of claim 26 for assaying for the presence of a -  $\alpha^{4.2}\alpha/\alpha\alpha$  genotype in a human individual.

29. The method of claim 26 wherein the marker amplicon to control amplicon ratio of step (c) is greater than zero.

30. A method of determining a hypertension-correlated genetic disorder in a hypertensive human individual comprising the steps of:

- (a) isolating DNA from said human individual;
- (b) assaying said DNA for a deletion relative to DNA of a normal human subject, said deletion comprising a reduction in the number of  $\alpha$ -globin genes in the genome of the hypertensive human individual; and
- (c) determining a presence of a hypertension-correlated genetic disorder

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in said individual from a presence of said deletion in said DNA.

31. A kit for determining the  $\alpha$ -globin genotype of a human individual comprising, in association:

5 a first pair of oligonucleotide primers for amplifying a first marker portion of a human chromosome 16, said first marker portion being absent from a human chromosome 16 having a single  $\alpha$ -gene deletion selected from the group consisting of an  $-\alpha^{3.7}$  deletion and a  $-\alpha^{4.2}$  deletion;

10 a second pair of oligonucleotide primers for amplifying a second marker portion of a human chromosome 16, said second marker portion being present in a human chromosome 16 having the single  $\alpha$ -gene deletion and being absent from a human chromosome 16 having an  $-\text{MED}$  deletion; and

a control pair of oligonucleotide primers for amplifying a control portion of human genomic DNA.

15 32. The kit of claim 31 wherein the single  $\alpha$ -gene deletion is an  $-\alpha^{3.7}$  deletion.

33. The kit of claim 32 further comprising a third pair of oligonucleotide primers for amplifying a third marker portion of a human chromosome 16, said third marker portion being absent from a human chromosome 16 having a  $-\alpha^{4.2}$  deletion.

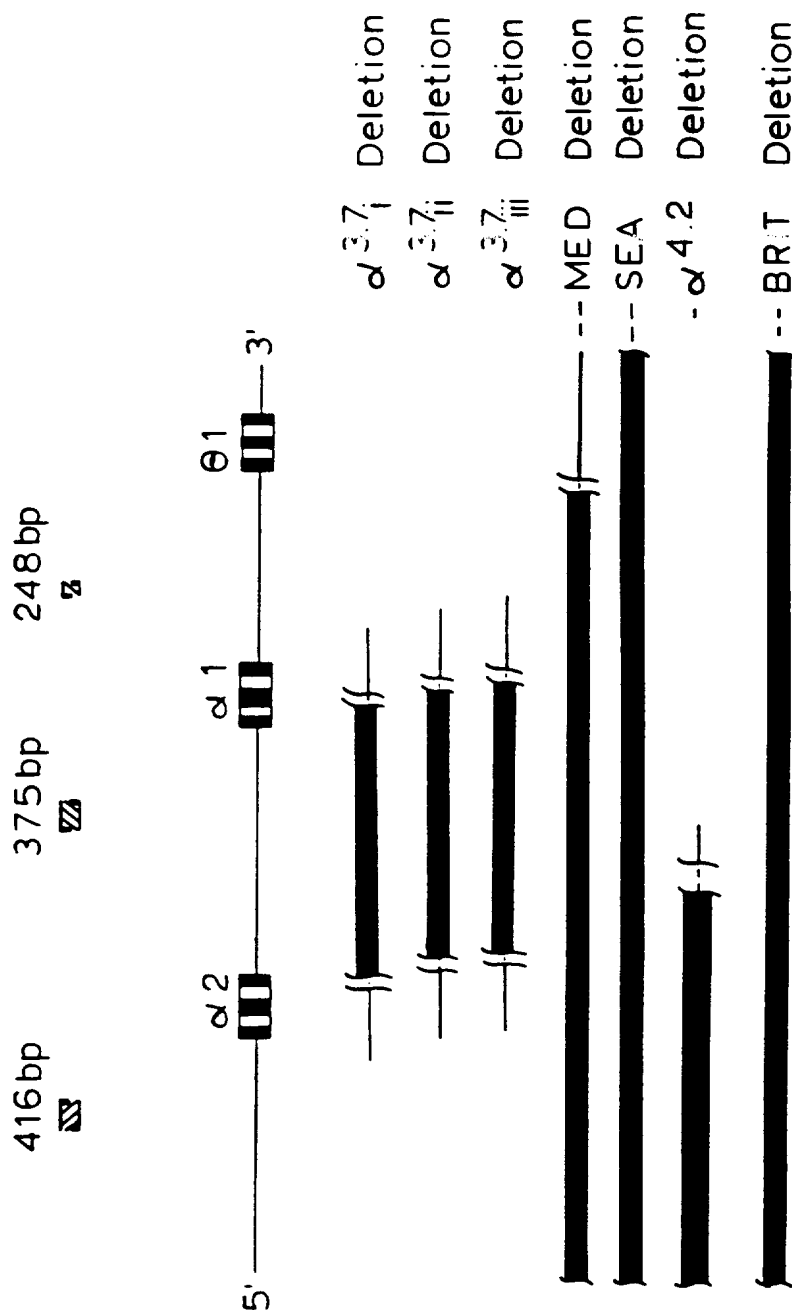
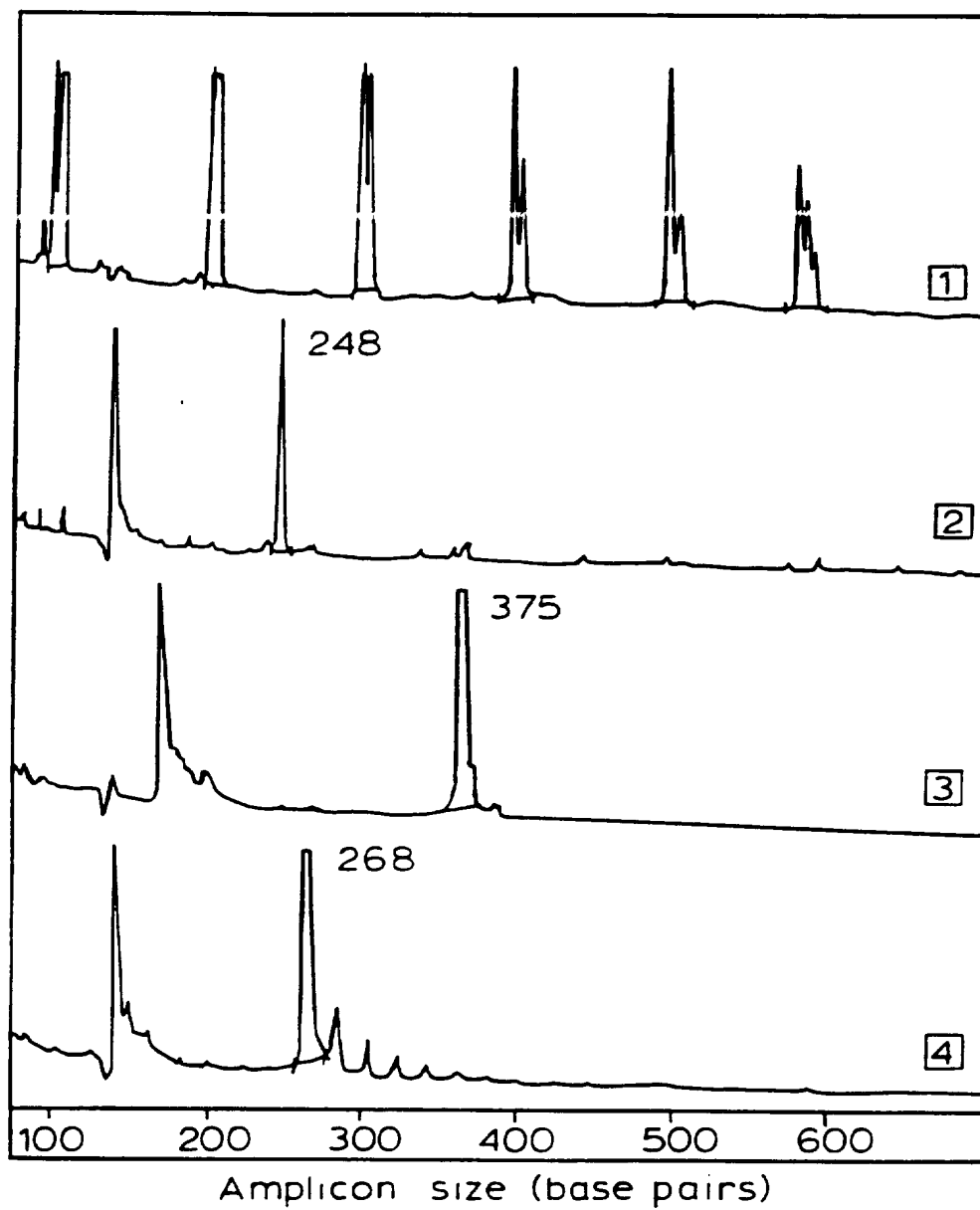
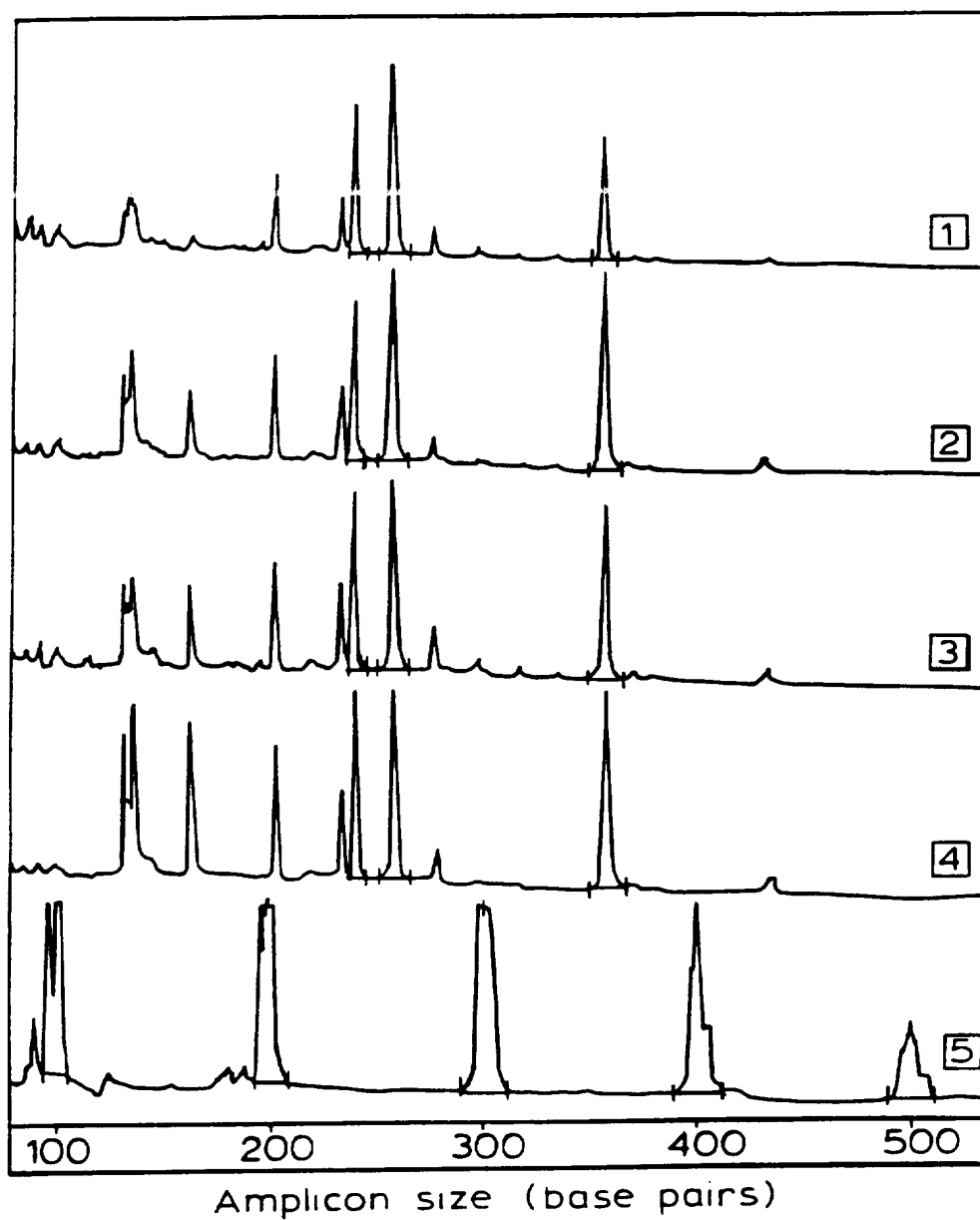


FIG. 1

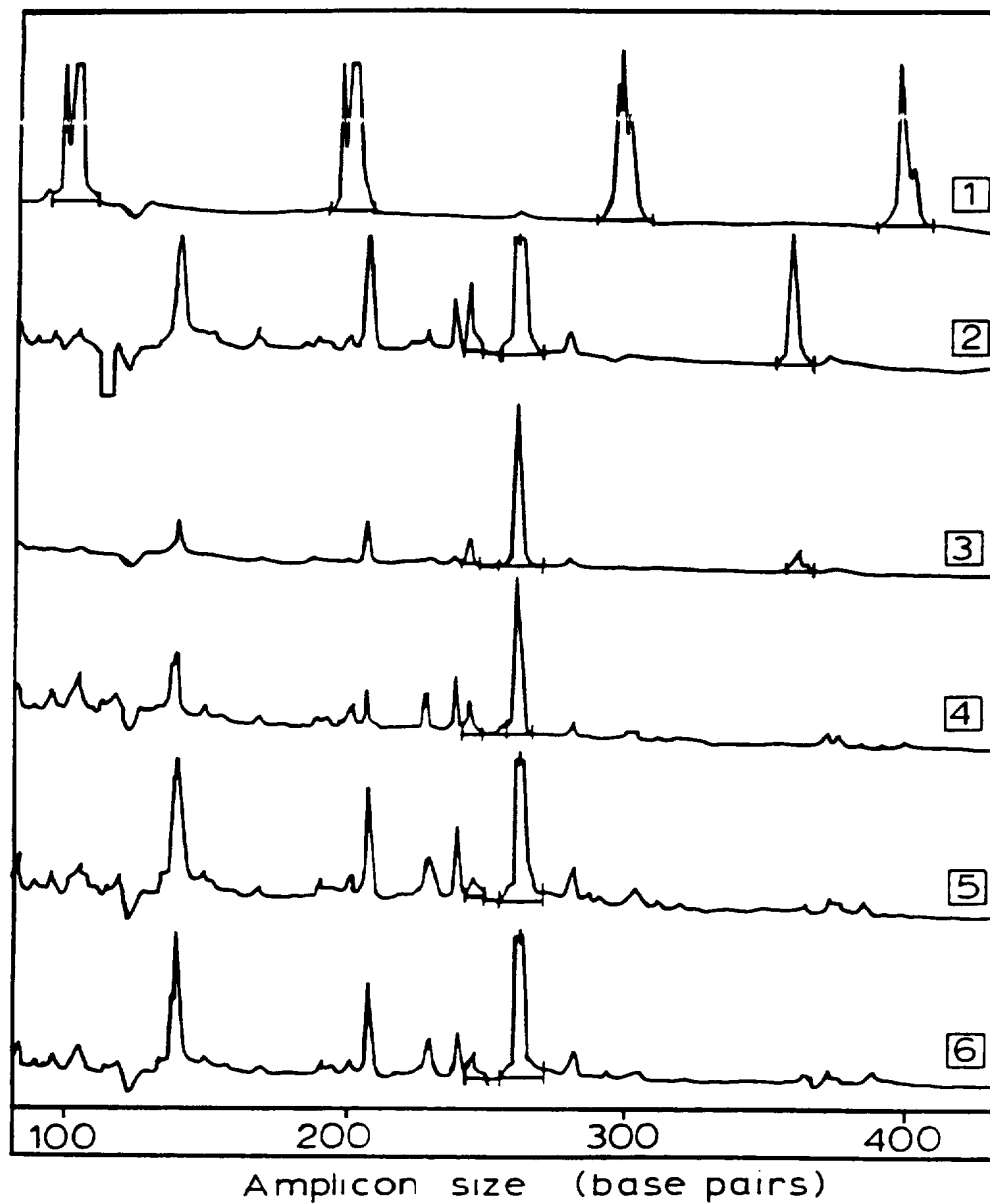
2 / 4

**FIG. 2**

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**FIG. 3**

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**FIG. 4**



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/17565

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CLINICAL CHEMISTRY, vol. 40, no. 12, October 1994, pages 2260-66, XP002024514 BOWIE L ET AL: "Detection of aphph-thalassemias by multiplex polymerase chain reaction" cited in the application	26-29, 31-33
A	see the whole document	1-6, 12-20, 22-25
X	--- AMERICAN JOURNAL OF HEMATOLOGY, vol. 45, April 1994, pages 279-82, XP000644343 ADAMS R ET AL: "Alpha thalassemia and stroke risk in Sickle anemia"	1-6, 21-25
A	see the whole document ---	26-33

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

7 February 1997

Date of mailing of the international search report

26.02.97

Name and mailing address of the ISA

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Authorized officer

Osborne, H

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/17565

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HEMOGLOBIN, vol. 19, no. 3 &amp; 4, 1995, pages 197-201, XP000616999 HARANO T ET AL: "HB Kurosaki: a new alpha chain variant found in japanese woman " see page 200, paragraph 3 ---</p>	1
A	<p>SEMINARS IN HEMATOLOGY, vol. 28, no. 3, July 1991, pages 202-8, XP000617394 POWARS D: "SICKLE CELL AMEMIA: BETA-S-GENE-CLUSTER HAPLOTYPES AS PROGNOSTIC INDICATORS OF VITAL ORGAN FUNCTION": see page 205 ---</p>	1
A	<p>DIABETES NUTRITION &amp; METABOLISM CLINICAL &amp; EXPERIMENTAL, vol. 4, no. 2, June 1994, pages 101-6, XP000617393 COSSU P ET AL: "HLA and 5' HVR insulin gene polymorphisms in thalassemia major patients with diabetes mellitus" see abstract ---</p>	1
A	<p>HEMOGLOBIN, vol. 16, no. 1 &amp; 2, 1992, pages 1-10, XP000617395 MIYASHITA H ET AL: "HB Kanagawa: A new aplha chain variant with an increased oxygen affinity " see abstract ---</p>	1
A	<p>WO,A,94 08048 (UNIVERSITY OF UTAH RESEARCH FOUNDATION) 14 April 1994 -----</p>	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/17565

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9408048	14-04-94	US-A- 5374525	20-12-94
		AU-B- 670990	08-08-96
		AU-A- 5725094	26-04-94
		CA-A- 2145415	14-04-94
		EP-A- 0665898	09-08-95
		JP-T- 8505043	04-06-96
		NZ-A- 259092	28-05-96
		US-A- 5589584	31-12-96
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